Journal of General Microbiology (1972), **73**, 439–446
Printed in Great Britain

Polygalacturonate Lyase of a Bacillus Species Associated with Increase in Permeability of Sitka Spruce (*Picea sitchensis*)

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(*Received* 21 *April; revised* 17 *July* 1972)

SUMMARY

The extracellular polygalacturonate lyase of a Bacillus species isolated from water-stored Sitka spruce (*Picea sitchensis*) was purified by ammonium sulphate fractionation and CM-cellulose chromatography. Its specific activity increased 59-fold and amylase, protease and xylanase activities were removed.

Degradation of the substrate was accompanied by a large decrease in viscosity, suggesting random attack. While calcium and strontium ions activated the enzyme, most divalent cations caused inhibition. Addition of EDTA resulted in complete inactivation. Enzyme activity was higher with acid soluble pectic acid than with sodium polypectate. The purified enzyme was stable over a wide pH range and had considerable resistance to thermal inactivation. This stability explains the enzyme's prolonged activity in water-stored spruce sapwood.

INTRODUCTION

During water storage of Sitka spruce (*Picea sitchensis*), bacterial degradation of the tori and bordered pit membranes results in a marked increase in the permeability of the wood to preservatives (Dunleavy & Fogarty, 1971*a*). A number of bacterial species were isolated from sap which had been aseptically pressed from the water-stored wood. When these isolates were examined for production of extracellular enzymes, two species possessed considerable pectinolytic activity (Fogarty & Ward, 1972*a*). This observation was significant in the light of reports that the pit membranes in sapwood contain considerable pectic material (Liese, 1970).

One of the pectinolytic organisms, a Bacillus species, which is being identified at this time, elaborates polygalacturonate lyase extracellularly. This enzyme cleaves pectic material by a transelimination process rather than hydrolysis. The enzyme is produced in liquid culture even when glucose is the sole carbon source in the growth medium (Ward & Fogarty, 1971). This organism also elaborates amylolytic, proteolytic and xylanase activities, although extracellular cellulolytic activity is negligible (Fogarty & Ward, 1972*a*). It increases the permeability of small sapwood blocks under laboratory conditions (Dunleavy & Fogarty, 1971*b*). Under such conditions polygalacturonate lyase activity is present in the blocks (W. M. Fogarty & O. P. Ward, unpublished). Polygalacturonate lyase and xylanase activities have also been detected in the expressed sap of water stored material (Fogarty & Ward, 1972*b*). Since a commercial pectinase can also cause an increase in the permeability of Sitka spruce sapwood, this Bacillus, mainly through its polygalacturonate lyase activity, probably plays an important role in increasing the permeability of Sitka spruce to preservatives.

In this present study, we report the isolation and purification of this polygalacturonate lyase and a number of its physico-chemical characteristics.

METHODS

Medium and culture conditions. The basal mineral medium consisted of $(NH_4)_2SO_4$, 2·0 g; K_2HPO_4, 5·0 g; KH_2PO_4, 1·0 g; KCl, 1·0 g; $MgCl_2.6H_2O$, 0·2 g; $CaCl_2.2H_2O$, 0·1 g; $MnSO_4.4H_2O$, 1·0 mg; $FeSO_4.7H_2O$, 0·5 mg. Glucose (1%) was normally used as carbon source; it was Seitz filtered and combined with the rest of the medium which had been autoclaved at 121 °C for 15 min. In one study, 1·0% sodium polypectate (Sigma Chemical Co.) was employed as sole carbon source. Media were dispensed in 500 ml vol. in 2 l Erlenmeyer flasks, inoculated with actively growing organisms at 27 °C and shaken at 150 rev./min in a New Brunswick Orbital Incubator Shaker (Model G25). Cultures were harvested by centrifugation after 48 h incubation; the supernatant fluid contained the crude enzyme with traces of amylase, protease and xylanase activities.

Substrates and enzyme assays. Acid-soluble pectic acid (ASPA) was prepared by heating 100 g of citrus pectin (Sigma Chemical Co.) in 2 l of 0·5 M-sulphuric acid at 100 °C for 1 h, followed by precipitation of ASPA from the filtrate with 2 vol. of ethanol (McCready & Seegmiller, 1954). Lyase activity was determined with a solution containing 0·2% ASPA, 0·001 M-calcium, and 0·05 M-tris-HCl buffer, pH 8·0. This substrate was prepared immediately before use. Samples (0·1 ml) of enzyme were added to 2·0 ml of substrate in all lyase assays. The rate of change in absorbance at 235 nm was measured in a 1 cm cell, using a Pye Unicam SP500 spectrophotometer equipped with a constant temperature unit. An enzyme unit is defined as the lyase activity releasing 1 μmol of product/min at 30 °C. A molar absorbtivity of 4600 M^{-1}/cm was used for this calculation (*Spectrophotometry nomenclature*, 1965).

Xylan (Nutritional Biochemical Co.), 1·0 g, was boiled in 25 ml deionized water with stirring for 5 min and 25 ml of 0·2 M-sodium phosphate buffer, pH 7·0 was then added. Xylanase activity was determined by measuring the increase in reducing groups associated with enzymatic degradation of xylan, using the dinitrosalicylic acid procedure (Bernfeld, 1955). Enzyme, 1·0 ml, was added to 1·0 ml of the 2·0% xylan substrate.

Amylose (Sigma Chemical Co.), 10·0%, was dissolved in 100 m dimethyl sulphoxide (W. M. Fogarty & P. J. Griffin unpublished) with constant stirring in a boiling water bath for 30 min. This solution was then stored at room temperature. Just before use, 10·0 ml of the stored amylose solution were added to 40·0 ml of 0·1 M-sodium phosphate buffer, pH 7·0. Enzyme, 1·0 ml, was added to 1·0 ml of this freshly prepared substrate and amylase activity was determined by measuring the increase in reducing groups produced. Both amylase and xylanase activity was evaluated in terms of μmol of glucose reducing equivalents/ml enzyme/min.

Casein, 0·5% dissolved in 0·1 M-sodium phosphate buffer, pH 7·0, was used as substrate in proteolysis determinations. Activity was estimated by measuring the solubilized tyrosine by the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951) after precipitation with 5·0% trichloroacetic acid.

All enzymes were assayed at 30 °C.

Purification procedure. The crude enzyme was precipitated with 90% ammonium sulphate and allowed to stand overnight at 4 °C. After centrifuging at 11 000 *g* for 1 h, the precipitate was resuspended in a minimum volume of deionized water and dialysed against several changes of 0·02 M-sodium acetate buffer, pH 6·0, over a period of 24 h. The dialysed fraction

was then applied to a CM-cellulose column (1·5 × 25 cm). In all separations carried out using CM-cellulose, the column was first equilibrated with 0·02 M-acetate buffer, pH 6·0. Elution was then carried out with 0·035 M-sodium phosphate buffer, pH 6·5; 5·0 ml fractions were collected and those containing the lyase activity were combined.

In preparation for gel filtration with the purified enzyme, the eluted active fraction was concentrated by ultrafiltration, using an Amicon cell containing a UM-2 Diaflo membrane. This membrane has a mol. wt retention limit of 1000 so the enzyme was therefore retained and concentrated. A 3·0 ml fraction of the concentrated enzyme was applied to a Sephadex G-75 column (1·5 × 80 cm) and eluted with 0·05 M-sodium phosphate buffer, pH 6·5. CM-cellulose gradient elution studies were carried out using both long (1·5 × 60 cm) and short (2·5 × 2·5 cm) columns and by eluting with a linear sodium phosphate gradient (0·001 to 0·1 M) at pH 6·5.

Viscosity reduction and bond hydrolysis. Viscosity reduction on hydrolysis of the substrate was determined using a capillary viscometer containing 0·5% sodium polypectate in 0·05 M-tris-HCl buffer, pH 8·0, containing 1 mM-Ca^{2+} as $CaCl_2$. The amount of bond cleavage after various times after adding enzyme was estimated by measuring the absorbancy change at 235 nm and relating this to the molar absorptivity value of 4600 M^{-1}/cm. Viscosity changes were examined by the method of Macmillan, Phaff & Vaughn (1964).

Activating and inhibiting effects. ASPA was treated with Amberlite resin, CG-120 (H^+ form), to remove contaminating cations. Divalent cations were added at 1 mM to 0·2% ASPA in 0·05 M-tris-HCl (pH 8·0). EDTA was tested in the presence of 1 mM-Ca^{2+}; the action of the chelating agents, 2,2,-dipyridyl and *o*-phenanthroline could not be assessed by this method since these had a high absorbance at 235 nm.

The relation between pH and activity was studied using both ASPA and sodium polypectate in 0·05 M-tris-HCl and Universal buffers (Campbell, 1955), with 1 mM-$CaCl_2$. To show any effect of the buffers themselves, lyase activity was determined with ASPA, 0·2% and $CaCl_2$, 1 mM over the range 0·01 to 0·2 M of tris-HCl, tris-glycine, Universal and sodium phosphate buffers at pH 8·0.

Tests of stability to pH and temperature. Portions (1·0 ml) of a range of Universal buffer solutions (0·1 M), pH 2·0 to 11·0, were added to 4·0 ml of enzyme, which had been dialysed against deionized water. Tubes were incubated at 4, 18, 30 °C for periods up to 24 h. Four vol. of 0·1 M-tris-HCl buffer, pH 7·0, were then added to each tube and 0·1 ml fractions were assayed in 2·0 ml of substrate to determine the residual activity.

Enzyme was diluted in Universal buffer at pH 5·0, 7·0 and 9·0, and also in deionized water. The mixtures were incubated at temperatures ranging from 0 to 65 °C for 24 h. After neutralization with 4 vol. of 0·1 M-tris-HCl, pH 7·0, the fractions were assayed for residual activity.

RESULTS

Enzyme purification. The crude enzyme preparation also contained protease, amylase and xylanase activities. One of the principal objectives was to free the lyase of these enzymes. When the dialysed 60 to 90% ammonium sulphate fraction was passed through a CM-cellulose column, both the lyase and the xylanase were bound, while most of the protein material was not retained. Phosphate gradient elution using both the long (1·5 × 60 cm) and the short (1·5 × 1·5 cm) columns gave similar elution patterns. The smaller column was too small for preparative work, but with it the elution peaks could be related more accurately to the phosphate concentration. While the lyase activity was eluted in a fairly sharp peak, Fig. 1 shows that this method failed to separate the lyase and xylanase enzymes. However

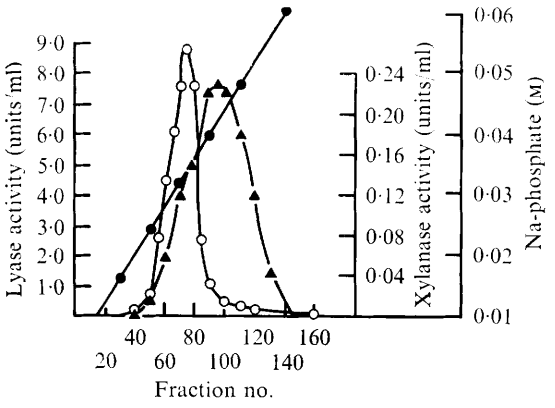

Fig. 1

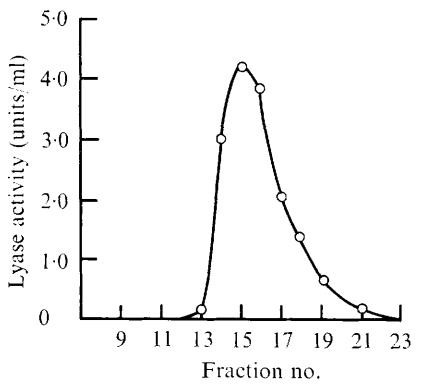

Fig. 2

Fig. 1. Elution of polygalacturonate lyase and xylanase from CM-cellulose with a linear sodium phosphate gradient. ○—○, Polygalacturonate lyase; ▲—▲, xylanase; ●—●, phosphate concentration (M).

Fig. 2. Elution of purified polygalacturonate lyase from Sephadex G-75. ○—○, Polygalacturonate lyase.

the sharpness of the lyase peak suggested that this enzyme contained only one component. Since glucose was normally used in the cultivation procedure, the elution pattern of lyase induced with sodium polypectate was observed and was similar: a single peak between fractions 60 to 95 (eluted by 0·03 to 0·052 M-sodium phosphate buffer) contained 94% of the total enzyme units recovered.

The slight difference in phosphate concentration at which these enzymes were maximally eluted (Fig. 1) from CM-cellulose suggested that gradient elution could be employed to separate them. Greatest elution of the lyase occurred at 0·035 M-sodium phosphate and the xylanase at 0·043 M. When the CM-cellulose column (1·5 × 25 cm) was eluted with 0·035 M-sodium phosphate, the lyase activity was eluted free of xylanase activity.

The protein contents of the active enzyme fractions were too dilute to be measured by the Lowry method or by direct absorbance at 280 nm. The fraction eluted with 0·035 M-phosphate was concentrated 15-fold by ultrafiltration through an Amicon UM-2 Diaflo membrane. This procedure retained 100% of the enzyme activity. The concentrated, purified enzyme was chromatographed on Sephadex G-75 (1·5 × 90 cm) from whence it emerged as one sharp peak (Fig. 2). Table 1 summarizes the results of the purification of the lyase. A 59-fold increase in specific activity was achieved with recovery of 44·8% of the original activity.

Relating reduction in viscosity to bond cleavage. Pectin and its demethylated product pectic acid are highly viscous polymers. Enzymes degrading these materials are classified on the basis of whether their attack is random or endwise. When a large reduction in viscosity (50%) occurs with only a small percentage of bond cleavage (2 to 3%) the enzyme is classified as an endotype. The enzyme discussed here caused a 50% reduction in relative viscosity when only 2·1% of the bonds were cleaved and therefore came into that category. Fig. 3 shows the relationship between viscosity reduction and bond cleavage as a function of time.

The effects of divalent cations and chelating agents. The effects of a number of divalent metal ions on activity were compared at 1 mM. Concentrations of cations above 1 mM were

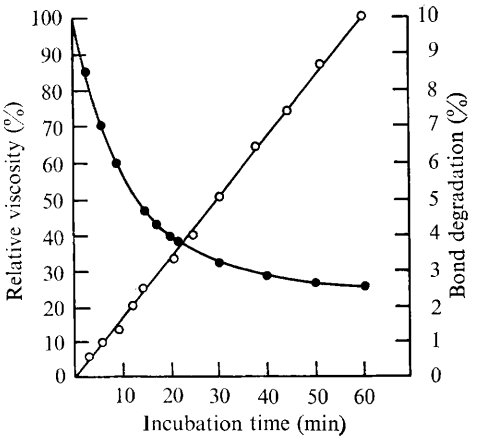

Fig. 3. The relation between viscosity decrease and bond cleavage as a function of the reaction time. ●—●, % relative viscosity; ○—○, % bond degradation.

Table 1. *Purification of the polygalacturonate lyase of a Bacillus species*

Stage	Volume (ml)	Activity (units/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg protein)	Yield (%)	Purification
Cell-free supernatant fluid	1000	9·14	9140	0·820	11·1	100·0	1·0
0 to 90% $(NH_4)_2SO_4$	62	104·60	6485	3·100	33·7	70·9	3·0
60 to 90% $(NH_4)_2SO_4$	115	47·90	5532	0·260	184·3	60·5	16·6
CM-cellulose chromatography	520	7·90	4108	0·012	654·9	44·8	59·0

not tested because they caused precipitation of substrate; the ASPA was freed of ions (see Methods). Ca^{2+} caused a 4·9-fold increase in activity; Sr^{2+} caused a 1·35-fold activation, while Cd^{2+} had a negligible effect. Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} all inhibited the control level of activity. Maximum activity was obtained at 0·4 mM-Ca^{2+}. EDTA (1·2 mM) inhibited lyase activity completely with 1 mM-Ca^{2+}.

Other activating and inhibitory effects. The purified enzyme was much more active towards ASPA than to sodium polypectate (Table 2). The pH optima for the enzyme in each of the assay media are also given. Activity was three times higher with ASPA in 0·05 M-tris-HCl than in Universal buffer. However, in Universal buffer the activity of the enzyme on sodium polypectate was 1·75 times that obtained in tris-HCl. When the effect of the concentration of the buffers on enzyme degradation of ASPA was examined, tris-HCl had an activating effect which was maximal at 0·05 M. Tris-glycine caused a 1·3-fold increase in activity above the level for tris-HCl alone. Universal and phosphate buffers both inhibited the enzyme; activity approached zero at a concentration of 0·1 M.

Stability of the purified lyase. The purified enzyme was found to be 100% stable for 24 h at 4, 18 and 30 °C over the pH range 3·4 to 8·8. Complete enzyme inactivation occurred at pH 9·9. At pH 2·5 complete inactivation occurred at 18 and 30, while at 4 °C, 28% of the original activity remained. At 4 °C complete inactivation was recorded at pH 2·0. The effect of temperature on enzyme stability is summarized in Table 3. The finding that the enzyme was 100% stable in deionized water at 30 °C justified our use of deionized water for dialysis in the purification procedure. Total activity was retained for 24 h at 37 °C at pH 9·0 and at

Table 2. *pH Optima and comparative activities of purified polygalacturonate lyase on ASPA and sodium polypectate*

Each substrate was prepared in both Universal and tris-HCl buffers (0·05 M). Lyase activity in each medium was measured at its optimum pH. For other details, see Methods.

Substrate	Buffer	Optimum pH	Lyase activity (units/ml)
ASPA	Tris-HCl	8·3	5·10
	Universal	7·4	1·65
Sodium polypectate	Tris-HCl	7·7	0·40
	Universal	7·5	0·70

Table 3. *Effect of temperature on stability of polygalacturonate lyase*

The enzyme was incubated at the temperatures indicated. After 24 h the percentage residual activity was estimated at 30°C after addition of 4 vol. of 0·1 M-tris-HCl buffer, pH 7·0. For other details, see Methods.

	Residual activity after 24 h incubation			
	Universal buffer			Deionized water
Incubating medium	pH 5·0	pH 7·0	pH 9·0	
Temperature (°C)	(%)	(%)	(%)	(%)
30	100	100	100	100
37	100	100	100	90
45	100	100	32	68
52	95	75	0	40
58	40	28	0	0
65	0	0	0	0

45°C between pH 5·0 and 7·0. While temperature inactivation was dependent on the incubating medium, complete denaturation had occurred in all media after 1 h at 65°C.

DISCUSSION

The single peak obtained by gradient elution from CM-cellulose, of the lyase of this Bacillus species suggests it contains only one component. In contrast, gradient elution of the polygalacturonate lyase from *Bacillus polymyxa* separated its activity into four components (Nagel & Wilson, 1970). Synthesis of most polygalacturonate lyases investigated to date has been induced by pectic substances (Nagel & Vaughn, 1961; Macmillan & Vaughn, 1964; Hasegawa & Nagel, 1966; Moran & Starr, 1969; Zucker & Hankin, 1970; Garibaldi & Bateman, 1971). The lyase activity of this Bacillus was highest when the organism was grown on glucose (Ward & Fogarty 1971). CM-cellulose gradient elution profiles, obtained after growth on (i) glucose and (ii) sodium polypectate, demonstrated that the lyases were chromatographically similar. Furthermore, the separation of the lyase from xylanase activity makes it possible to evaluate their individual contributions to the increase in permeability of water-stored wood.

This enzyme can be classified as an endopolygalacturonate lyase, similar to the lyases of other Bacillus species (Nagel & Vaughn, 1961; Hasegawa & Nagel, 1966), *Erwinia carotovora* (Moran, Nasuno & Starr, 1968), *Xanthomonas campestris* (Nasuno & Starr, 1967) and *Pseudomonas fluorescens* (Fuchs, 1965). In contrast, the lyases of *Clostridium multifermentans* (Macmillan *et al.* 1964) and *E. aroideae* (Okamoto, Hatanaka & Ozawa, 1964) attack in an endwise manner.

Like other lyases, the enzyme is activated by Ca^{2+}. In contrast to this, the hydrolytic polygalacturonases do not require Ca^{2+} (Voragen & Pilnik, 1970). In agreement with other reports (Nagel & Vaughn, 1961; Macmillan & Vaughn, 1963; Hasegawa & Nagel, 1966) it has been shown that some lyase activity is present in the absence of added Ca^{2+}. Failure to remove Ca^{2+} associated with the ASPA may be responsible for this activity. If EDTA inhibits the enzyme solely by binding divalent cations, then the divalent cation is an absolute requirement for lyase activity.

Effects of other ions on enzyme activity may depend on whether the enzyme causes random or endwise cleavage. All divalent ions other than Zn^{2+} stimulate the exopolygalacturonate lyase of *Clostridium multifermentans* to some degree (Macmillan & Vaughn, 1964). Endo-polygalacturonate lyases have only shown activation with Ca^{2+} or Sr^{2+} (Voragen & Pilnik, 1970). This present study also illustrates that other divalent cations actually inhibit enzyme activity.

The purified lyase shows considerable resistance to denaturation. It has been shown that both polygalacturonate lyase activity (Fogarty & Ward, 1972*b*) and also permeability increase (Dunleavy & Fogarty, 1971*a*, *b*) continues in water-stored Sitka spruce for weeks after bacterial numbers have decreased. The stability properties of this enzyme, therefore, explain the prolonged action of polygalacturonate lyase in the water-stored wood.

The authors wish to thank the Forest and Wildlife Service, Department of Lands, Dublin 2, for a grant in support of this project.

REFERENCES

BERNFELD, P. (1955). Amylases α and β. *Methods in Enzymology* **1**, 149–158.

CAMPBELL, L. L. (1955). Purification and properties of an α-amylase from facultative thermophilic bacteria. *Archives of Biochemistry and Biophysics* **54**, 154–161.

DUNLEAVY, J. A. & FOGARTY, W. M. (1971*a*). The preservation of spruce poles using a biological pretreatment. *Proceedings of the British Wood Preserving Association 21st Annual Convention, Cambridge*, pp. 1–24.

DUNLEAVY, J. A. & FOGARTY, W. M. (1971*b*). The effects of ponding on the permeability of Sitka spruce (*Picea sitchensis*). *Abstracts of 2nd International Biodeterioration Symposium, Lunteren, The Netherlands*, **16**, 10.

FOGARTY, W. M. & WARD, O. P. (1972*a*). Enzyme production by bacteria isolated from water stored Sitka spruce (*Picea sitchensis*). *Journal of Applied Bacteriology* (in the Press).

FOGARTY, W. M. & WARD, O. P. (1972*b*). Microbial enzymes associated with increased permeability of Sitka spruce (*Picea sitchensis*) to preservatives: their detection in the expressed sap. *Journal of General Microbiology* **71**, xi–xii.

FUCHS, A. (1965). The trans-eliminative breakdown of Na-polygalacturonate by *Pseudomonas fluorescens*. *Antonie Van Leeuwenhoek* **31**, 323–340.

GARIBALDI, A. & BATEMAN, D. F. (1971). Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. *Physiological Plant Pathology* **1**, 25–40.

HASEGAWA, S. & NAGEL, C. W. (1966). A new pectic transeliminase produced exocellularly by a bacillus. *Journal of Food Science* **31**, 838–845.

LIESE, W. (1970). Ultrastructural aspects of woody tissue disintegration. *Annual Review of Phytopathology* **8**, 231–258.

LOWRY, O., ROSEBROUGH, N., FARR, A. & RANDALL, R. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

MCCREADY, R. M. & SEEGMILLER, C. G. (1954). Action of pectic enzymes on oligogalacturonic acids and some of their derivatives. *Archives of Biochemistry and Biophysics* **50**, 440–450.

MACMILLAN, J. D., PHAFF, H. J. & VAUGHN, R. H. (1964). The pattern of action of an exopolygalacturonic acid-trans-eliminase from *Clostridium multifermentans*. *Biochemistry* **3**, 572–578.

MACMILLAN, J. D. & VAUGHN, R. H. (1964). Purification and properties of a polygalacturonic acid-trans-eliminase produced by *Clostridium multifermentans*. *Biochemistry* **3**, 564–572.

MORAN, F., NASUNO, S. & STARR, M. P. (1968). Extracellular and intracellular polygalacturonic acid trans-eliminases of *Erwinia carotovora*. *Archives of Biochemistry and Biophysics* **123**, 298–306.

MORAN, F. & STARR, M. P. (1969). Metabolic regulation of polygalacturonic acid trans-eliminase in *Erwinia*. *European Journal of Biochemistry* **11**, 291–295.

NAGEL, C. W. & VAUGHN, R. H. (1961). The characteristics of a polygalacturonase produced by *Bacillus polymyxa*. *Archives of Biochemistry and Biophysics* **93**, 344–352.

NAGEL, C. W. & WILSON, T. M. (1970). Pectic acid lyases of *Bacillus polymyxa*. *Applied Microbiology* **20**, 374–383.

NASUNO, S. & STARR, M. P. (1967). Polygalacturonic acid trans-eliminase of *Xanthomonas campestris*. *Biochemical Journal* **104**, 178–185.

OKAMOTO, K., HATANAKA, C. & OZAWA, J. (1964). The mechanism of action of the saccharifying pectate trans-eliminase. *Berichte des Ohara Instituts Fuer Landwirtschaftliche Biologie, Okayama Universitaet, Japan* **12**, 115–119.

Spectrophotometry nomenclature (1965). *Analytical Chemistry* **37**, 1814.

VORAGEN, A. G. J. & PILNIK, W. (1970). Pektindepolymerasen: I Einteilong der Pektinsauredepolymerasen Nach Ihrer Wirkung auf Oligomere Galakturonsauren. *Zeitschrift für Lebensmittel-untersuchung und Forschung* **124**, 346–359.

WARD, O. P. & FOGARTY, W. M. (1971). Extracellular enzymes of a *Bacillus* sp. associated with increased permeability in Sitka spruce (*Picea sitchensis*). *Biochemical Journal* **125**, 109 P–110 P.

ZUCKER, M. & HANKIN, L. (1970). Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. *Journal of Bacteriology* **104**, 13–18.