Production of extracellular pectic enzymes from Erwinia carotovora (Isolate 14) incubated under aerobic and anaerobic conditions.

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PRODUCTION OF EXTRACELLULAR PECTIC ENZYMES
FROM ERWINIA CAROTOVORA (ISOLATE 14) INCUBATED UNDER
AEROBIC AND ANAEROBIC CONDITIONS

A Thesis Presented
By
STEPHEN JOHN NOVAK

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
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Department of Plant Pathology
PRODUCTION OF EXTRACELLULAR PECTIC ENZYMES
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May 1981
To my parents and Cathy
whose love and support has
made this work possible.
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ABSTRACT

Cultures of *Erwinia carotovora* isolate 14 were incubated under aerobic or anaerobic conditions at either 24 or 30°C. Cultures incubated under anaerobic conditions exhibited reduced growth when compared to those cultures incubated aerobically. However, the extracellular protein concentrations of these incubation treatments were similar. The level of production of extracellular endopectate lyase (PL) was dependent on temperature more than on any other environmental parameter. Highest levels of PL were produced in cultures incubated at 30°C either aerobically or anaerobically. The level of production of extracellular endopolygalacturonase (PG) were dependent on the availability of oxygen. Highest levels of PG production were observed for those cultures incubated at 24 or 30°C under aerobic conditions. Extracellular pectic enzyme activity values were adjusted to a level of production which would have been achieved by a $10^8$ cells/ml culture. As a result, the production levels of extracellular pectic enzymes from EC14 were enormously increased in the anaerobically incubated cultures. Two possible mechanisms which may explain this phenomena are suggested.
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CHAPTER I
Introduction

A large and very diverse group of pathogens have been found to possess the ability to degrade the constituents of the cell walls of higher plants. These organisms utilize degradative enzymes in order to gain access into a host and have been implicated in a wide variety of plant diseases (5, 6, 7, 8, 21, 32, 35, 44, 56, 60), including soft rots, dry rots, wilts, blight and leaf spots. By far, the most important of these enzymes in terms of the degree of tissue damaged and dollars lost are the pectic enzymes (1, 5, 6, 7, 8, 9, 12, 32, 33, 34, 44, 51, 52, 55, 57).

Erwinia carotovora is a phytopathogenic bacterium which possesses the ability to produce a wide variety of pectic enzymes (7, 8, 23, 30, 31, 32, 36). Bacterial soft rot of potato tubers in storage and transit has been attributed most often to Erwinia spp. (10, 49, 55, 61). Typically, in the soft rot disease of potato tubers caused by E. carotovora, symptoms require the presence of sufficient levels of pectic enzymes and follow a pattern of electrolyte loss, tissue maceration, and cellular death (32, 55, 57). Several investigators have demonstrated that the production of extracellular levels of a pectic enzyme, endo-polygalacturonate trans-eliminase (PGTE) is integrally related to symptom initiation and soft rot progression (32, 57). An avirulent mutant strain of E. carotovora was produced by nitrosoquandine (NTG) treatment and was found to be deficient in the ability to synthesize extracellular pectic enzymes and thus unable to cause
disease in a potential host (9).

Erwinia carotovora (Jones) Holland isolate 14 (EC14) has been shown to produce two extracellular pectic enzymes, endopectate lyase (PL) and endopolygalacturonase (PG) (32, 51, 52, 57). These enzymes, in either crude or purified form, induce soft rot conditions in plant tissues. Subsequently, an intracellular pectin depolymerase complex was isolated and characterized from this isolate (51, 52). This complex consisted of four enzymes which were designated PDI through IV. PDI was shown to possess dual activities and was determined to be identical to the extracellular enzymes produced by this bacterium. The regulatory mechanism(s) of pectic enzyme synthesis has also been partially characterized for this isolate (19, 34).

Soft rot development of potato tubers has been found to depend on a number of factors. Two of these factors are the environmental conditions surrounding the tuber, and the types and numbers of pectolytic microorganisms in or on the tuber (10, 26). The most critical of the environmental factors in determining soft rot development are the presence of a film of water around the tuber and the depletion of oxygen within the tuber (11, 15, 16, 25, 26, 27, 38, 44, 45, 50, 64).

To date little work has been conducted to determine the effects the environmental conditions have on the synthesis of pectic enzymes and how this is related to the rotting of potato tubers. The aim of this study was to determine the effects of temperature and aerobic versus anaerobic conditions on PL and PG production, in EC14, as it relates to soft rot progression.
CHAPTER II

Literature Review

Bacterial soft rot of potatoes in storage or transit has been attributed most often to *Erwinia* spp.- particularly *E. carotovora* var. *carotovora* (Ecc) and *E. carotovora* var. *atroseptica* (Eca) (10, 49, 55, 61). Other pectolytic bacteria have also been found to be present and may contribute to the soft rot condition. These include: *Pseudomonas* spp., *Bacillus* spp., *Clostridium* spp., *Aerobacter* spp. and *Flavobacterium* spp. (44, 45, 46, 49).

The exact estimate of losses due to soft rot is difficult to determine, but some figures range as high as $50 to $100 x 10^6 annually on a worldwide basis (44). These figures will vary depending on the climatic conditions and the value of the crop which is being attacked.

A number of factors may influence the initiation and spread of decay in potato tubers; these include: (1) environmental conditions, particularly temperature, relative humidity, aeration and the presence or absence of a film of water on the surface of the tuber; (2) injury to the tuber during harvest or handling; (3) the amount of soil or plant debris brought into storage with the tubers; (4) the type and amount of chemicals applied to the plant or tuber during the growing season and in storage; (5) the types and numbers of microorganisms, particularly soft-rotting bacteria in or on the tuber; and (6) the inherent physiological condition and rate of metabolism of the tuber (10, 26).
The environmental factors associated with soft rot disease development have been studied extensively by many investigators (15, 16, 25, 27, 38, 44, 45, 49, 64). It has been determined that the presence of high relative humidity and temperatures greatly increase the development of soft rot symptoms. Tuber decay in rail shipments from the State of Washington accounted for 63% of the total tuber defects from 1965 to 1969 (15). Tubers which were kept dry and stored for 11 days over ranges of 10-21°C and 2-20.5% O₂ levels were found to be free of any soft rot development. Tubers which were partially immersed in water did not show rot development when cooled to 4.4°C in less than 24 hours after harvest and held for 8 days at 4 to 20.5% O₂ levels. When tubers were immersed in water at 10°C or higher after harvest and held for 8 days at 4 to 20.5% O₂ levels soft rot symptom developed. Tubers which had been partially immersed and incubated for 1 day at 21°C developed rot in a low oxygen (4%) atmosphere when later stored for 8 days at 4.4°C (15).

However, the major environmental determinants involved in soft rot development are probably the presence of a film of water on the tuber surface and the depletion of oxygen within the tuber (11, 15, 16, 25, 26, 27, 38, 44, 45, 50, 64). This depletion of oxygen may be the result of a number of factors. When mature tubers are covered with a persistent film of water with an average thickness of about $3 \times 10^{-2}$ mm anaerobic conditions will occur after 6½ hours at 10°C or 2½ hours at 21°C (11). It has been determined that restricted aeration in closed containers, during transit, resulted in an increase in the incidence of tuber infection and decay as respiratory gases accumulated around
A number of investigators have shown that low concentrations of O\(_2\) will increase the rate of rotting of potato tubers (15, 16, 25, 27, 38, 44, 45, 50). The susceptibility of King Edward potatoes to soft rot was determined using varying concentrations of oxygen and carbon dioxide (27). Tubers were wounded, inoculated with *E. carotovora* var. *atroseptica* and stored at 10°C and 100% relative humidity in controlled concentrations of oxygen and carbon dioxide for up to 18 days. Extensive, soft spreading rots were produced in potatoes stored at 5% O\(_2\) + 16% CO\(_2\), in 1% O\(_2\) + 20% CO\(_2\), in 1% O\(_2\) and in 1% N\(_2\), the most extensive rots occurred at the 1% O\(_2\) and N\(_2\) atmospheres (27).

When 10 ml of six inoculum levels (1x10\(^6\) to 5x10\(^9\)) were injected into potato tubers it was determined that the bacteria acted independently and that tubers were equally susceptible to decay. Greater amounts of decay were recorded at the 2% O\(_2\) level as compared to the 6 at 10% O\(_2\) levels (16).

The increased rate of rot which occurs at low O\(_2\) concentrations is believed by some to be an inhibition of the resistance mechanisms of the tuber (16, 25, 49, 64). A decrease in the rate of suberization and periderm formation, as well as the wound healing response, of potato tubers is observed as a result of low (½ to 1%) levels of O\(_2\) (25, 64). These conditions have been found to occur during transcontinental rail and truck shipments of fresh fruits and vegetables (25).

A possible sequence of events occurring under wet anaerobic conditions has been suggested (45). It is believed that when excess water
is absorbed the lenticels are opened; an oxygen deficiency will affect cell membrane integrity causing leakage of water and solutes from turgid cells. This can establish a continuous liquid phase between the cortex of the tuber and the lenticel, thereby allowing microorganisms in the lenticels to penetrate deeper into the tuber via this liquid stream.

The combination of the leakage of water and solutes from the tuber with the decrease in the resistance of the tuber under anaerobic conditions enables pectolytic bacteria to proliferate and soft rot disease to develop.

The cell wall provides the initial barrier that a possible pathogen must overcome if it is to invade a host. For this reason a discussion on the cell wall and its components is relevant. The major components of the cell wall are pectic polysaccharides, cellulose, hemicellulose, structural glycoproteins and lignin (2, 7, 20, 24, 32, 48). The cell wall can be viewed as a two phase system — the first consisting of a dispersed phase of cellulose microfibrils and the second being a complex continuous matrix (7, 32). The cell wall has been divided into three functional-structural regions, the middle lamella, the primary cell wall and the secondary cell wall (2, 7, 32). The primary cell wall of various plants are probably similar in structure and function (32). It is the first wall region formed with definite organisation and consists mainly of pectic substances, cellulose and hemicelluloses and serves to support and protect the cell protoplast (7).
The secondary cell wall is added after cell elongation is completed (7). A great deal of variation occurs in the secondary cell wall from plant to plant and tissue to tissue (54). The principle component is cellulose, but pectic substances, hemicelluloses and lignin are also found in this region.

The middle lamella is the area where adjacent cells are linked into a tissue system (54). In herbaceous tissues the middle lamella is mainly composed of pectic substances. In lignified tissues, lignin is the major component of the middle lamella (32). A hydroxyproline rich glycoprotein has also been identified in plant cell walls (20, 24, 40). Although its occurrence and function are not well known it is thought that this may add to the structural integrity of the pectic component.

The major component of the pectic portion of the primary cell wall and the middle lamella is a high molecular weight polymer consisting of a backbone of α-1, 4-linked D-galacturonopyranose interspersed with 1, 2-linked rhamnopyranose (20, 54). Polymers of neutral sugars are also present which may serve as a bridge between the rhamnogalacturonan and hemicellulosic wall components. The carboxyl group of uronic acid may possess a certain degree of methylation (8). If over 75% of the uronide residues are methylated the polymer is referred to as pectin. Those polymers with a lower degree of methylation are referred to as pectinic acid. When no uronide residues are esterified with methyl groups this molecule is known as pectic acid. The uronic acid residues may also be acetylated at positions 2 and 3.
In some cases pathogens will utilize primarily mechanical means to gain access into a host. A large majority of pathogens, however, utilize a wide variety of enzymes which are capable of degrading cell wall constituents. These organisms possess the ability to produce cell wall degrading enzymes and have been implicated in a wide variety of plant diseases (5, 6, 7, 8, 21, 32, 35, 44, 56, 60). These include soft rots, dry rots, wilts, blights and leaf spots. Of this variety of cell wall degrading enzymes available in the arsenal of pathogenic organisms, probably the most important in terms of the degree of tissue damaged and dollars lost, are the pectic enzymes (1, 2, 5, 6, 7, 8, 9, 12, 32, 33, 43, 44, 51, 52, 55, 57).

Pectic enzymes can be classified by several criteria: (1) the mechanisms by which the 1, 4 glycosidic linkage is cleaved (hydrolytic or lytic); (2) the optimal activity the enzyme has for a substrate; and (3) whether cleavage of the substrate occurs in a random (endo) or terminal (exo) manner (8, 48).

Hydrolase enzymes, such as pectin esterase and polygalacturonase, have an acidic pH optimum (pH 4.0 to 6.5) and do not require metal ions to exhibit activity (5, 6, 7, 8, 42, 46, 51, 52). In some cases fungal polygalacturonases have been reported to be inhibited by calcium ions (5). Pectin esterase hydrolyses the esterified methyl groups of the galacturonosyl chain producing uronic acid groups and methanol as reaction products. The activity of pectin methylesterase can be determined by measuring the amount of liberated methanol (23). The pectin chain length is not affected by this enzyme although removal of the methyl
group makes pectin more vulnerable to attack by other polysaccharide degrading enzymes (7, 8, 32).

Polygalacturonase has been shown to have a specificity for non-methoxylated polygalacturonic acid (7, 8, 32). Endopolygalacturonases result in several size products (diner to possible pentamer) while exoforms of this enzyme release di-galacturonic acid from the polymer (18). The endo-enzymes have been shown to induce soft rot symptoms when isolated from the organism and either purified or used directly (32, 55).

Trans-eliminase or lyase enzymes break the glycosidic linkage of the pectin polymer at carbon 4 and simultaneously eliminates the H+ from carbon 5. This results in oligouronides which terminate in a modified carbon 4, 5 unsaturated galacturonyl unit and a loss in viscosity (7, 8, 32). This loss of viscosity and the formation of the double bond are used to assay lyase activities (5, 6, 47, 62). Polygalacturonic acid rather than methylated pectin appears to be preferred substrate. Some lyases have been shown to attack pectin, depending on the degree of methylation. The pH optimum for lyase enzymes is in the alkaline range (pH 7.5 to 9.5) (7, 8, 32). The lyase activity is stimulated by specific concentrations of calcium, although higher or lower levels of calcium can result in inhibition of enzyme activity (3, 28, 30). The presence of a chelating agent, such as ethylene diamine tetraacetic acid (EDTA) will also inhibit enzyme activity (32, 48). Other divalent cations have had no effect on lyase activity in E. carotovora, however, the activity of a lyase from Clostridium
**multifermentens** was greatly enhanced by other divalent cations such as strontium and magnesium (28).

Phytopathogenic *Erwinia* species induce soft rot to develop because they are capable of producing a variety of pectic enzymes, including intra and extracellular forms of lyase and hydrolase enzymes (30, 33, 36, 51, 52). An *E. carotovora* isolate from soft rot spoilage in a shipment of green peppers was grown in a semisynthetic pectin medium and found to produce extracellular pectinesterase and polygalacturonase (23). The polygalacturonase had a wide pH range extending from 3.9 to 7.9, with a broad optimum around pH 5.8 as measured by the release of free reducing groups from the substrate. It was later shown that galacturonic acid is converted by isomerization and reduction into D-hexonic acid which is further metabolized into triose-phosphate and pyruvic acid (22). The pyruvic acid is then utilized in the tricarboxylic acid cycle.

A polygalacturonic trans-eliminase (PGTE) was later isolated from isolate 153 of *E. carotovora* (52). This enzyme had a pH optimum of 8.5, was stimulated by the addition of up to 0.001 MCaCl₂ and was inhibited by 3x10⁻⁵ M EDTA, although activity was restored upon the addition of 0.001M CaCl₂. Under the investigators assay conditions a temperature optimum of 50°C was observed.

This same group successfully purified a polygalacturonase from polygalacturonic acid trans-eliminase which was produced in culture by isolate 153 of *E. carotovora* (36). This enzyme was purified 56 fold by means of acetone precipitation, acetate buffer extraction, and
two successive treatments by column chromatography on carboxymethyl cellulose. This enzyme was shown to have a pH optimum between 5.2 and 5.4. The major end products were determined to be mono- and digalacturonic acid and digalacturonic acid was not hydrolyzed further.

Isolate 153 was also shown to have an intracellular polygalacturonic acid trans-eliminase which had properties similar to the extracellular polygalacturonic trans-eliminase (30). These trans-eliminases had a pH optimum of 8.5, was stimulated by calcium and had an endo mode of action. The major end product was determined to be unsaturated digalacturonic acid.

An oligogalacturonide trans-eliminase (OGTE) was later purified from isolate 153 of E. carotovora (31). This enzyme degraded D-galacturonic acid into two molecules of deoxyketuronic acid, indicating an eliminase (lyase) reaction. OGTE attacks unsaturated digalacturonic acid at 400 times the rate that polygalacturonic acid is attacked. The rate of attack of oligouronides varies inversely with the chain length.

An endo-PGTE was purified from E. carotovora (Jones) Holland isolate 14, a soft rot bacterium, which induced electrolyte loss, tissue maceration and cellular death of potato tuber (33). This PGTE was purified 295-fold from crude culture filtrate by ammonia sulfate precipitation, column chromatography on DEAE-cellulose, and isoelectric point focusing. This enzyme was shown to have a pH optimum of 8.5 and required calcium for activity. An endo-PGTE, which was purified through isoelectric focusing was purified from this same bacterium and was found to macerate potato and cucumber tissue disks and induce
leakage of $^{86}$Rb from potato disks (57). Death of host cells was shown to be caused by the inability of the cell wall to support the protoplast, when exposed to high water potentials, which results in cell lysis (1, 17).

An intracellular pectic enzyme complex of _E. carotovora_ was discovered, purified through isoelectric focusing, and partially characterized (51, 52). This complex was comprised of at least four pectate depolymerases which were designated PDI, PDII, PDIII, and PDIV. PDI had an isoelectric point (PI) of 9.4 and possessed the ability to depolymerize a sodium polypectate (NaPP) substrate in both an endo-PGTE manner at pH 8.5 in the presence of Ca$^{++}$, and in an endo-PG manner at pH 6.0 in the presence or absence of divalent cations. PDI is the only pectic enzyme that is found outside the cell; the extracellular form has been designated PDIa. PDIa was found in a culture filtrate of nutrient media as well as in rotted potato tubers. When purified PDI was reacted with potato tuber disks at pH 6.0 or 8.5 tissue maceration and cellular death occurred.

Further work has led to a partial characterization of the regulatory mechanism governing lyase synthesis in _E. carotovora_ isolate 14 (19, 34, 65). As had been demonstrated earlier the synthesis of lyase is inducable and subject to catabolite repression after cells growing in NaPP were supplemented with glucose (19). Exogenously supplied adenosine 3'-5' cyclic monophosphate (cAMP) reversed repression of intracellular PGTE activity. A decrease in cAMP was correlated with glucose repression of PGTE activity. As PGTE activity increased in
induced cultures, the specific concentration of cAMP increased. Various analogues of cAMP were unsuccessful in reversing glucose repression of PGTE. "Self" catabolite repression of pectate lyase (16) activity in *E. carotovora* strain ECl was observed when high concentrations of the inducer accumulated in the medium (59). Repression of the induction by high concentration of inducer was relieved by consumption of the inducer and by the addition of $2 \times 10^{-3} \text{M cAMP}$. Subsequently, a cAMP-deficient mutant of *E. carotovora* was isolated from a nitrosourea-treated B-galactosodase constitutive strain (34). This mutant was unable to use $\alpha$ lactose, L-arabinose, D-galactose, L-rhamnose, D-xylose, raffinose, D-cellobiose, glycerol and sodium polypectate as a sole carbon source unless exogenous cAMP was supplied. The synthesis of PGTE was found to be under the direct control of the cAMP regulatory mechanism. Only low levels of PGTE were detected when this mutant was grown on a casein hydrolysate-minimal salts medium, even in the presence of the inducer. PGTE levels would increase to those of the parent strain when NaPP and cAMP were added to the medium. This is a similar regulatory mechanism to that found in *Escherichia coli*.

Pectic acid was added to a minimal medium in which strain ECl of *E. carotovora* was growing (58). Time course samples were extracted, washed, sonicated and assayed. An increase in Pectic Acid lyase (PAL) was observed, but a long lag phase was evident before the onset of induction. This suggested that pectic acid was not inducing directly. The true inducer was sought in time-course induction experiments. A rapid induction of PAL activity was observed specifically when $2 \times 10^{-3} \text{M}$
purified unsaturated digalacturonic acid was added to a minimal media as the sole carbon source. No induction was observed after the addition of di-galacturonic acid, which is the major product of the cleavage of pectic acid by *E. carotovora* PG enzymes.

Both unsaturated and saturated digalacturonic acid (UDG and DG) or their metabolites, have been found to induce endopectate lyase in *E. chrysantheni* (13, 14). Oligogalacturonate lyase (OGL) converts MDG and UDG into 4-deoxy-5 keturonic acid (DK) which was found to be a highly effective inducer of PL synthesis. These investigators postulate that DK (or its precursor) is the actual inducer and that OGL is the primary generator of the apparent PL inducer and its high rate of activity on UDG promotes self-catabolite repression of PL. An OGL-deficient mutant was poorly induced unless supplied with deoxyketuronic acids or a substrate that could be converted by another intracellular enzyme to DK (13).

To date little work has been conducted on the effect environmental conditions have on the synthesis of pectic enzymes and how this is related to the rotting of potato tubers.

The growth rate *in vitro* of *Erwinia* spp. was reduced when cells were grown in low oxygen atmospheres (1 to 3%) (29, 63). The mean growth rate of *E. carotovora* was reduced to 50-64% of that in air at 3% O₂ levels. At 0% O₂ growth was between 3 and 12.5% of that in air. In the absence of CO₂ *Erwinia* spp. did not grow at any concentrations of O₂ during a 24-hour period (63).

With reduced growth rates decay develops because only small
conditions on endo-PG and endo PL production, from \textit{E. carotovora} var. \textit{carotovora}, as it related to soft rot progression. Most investigators believe that PL is the primary cause of tissue maceration in soft-rotted tubers. However, the observation that rotted tubers tend to have a lower pH than normal (near 6.0 the optimal pH for PG activity) and recent work, in controlled environmental chambers, with whole tubers under anaerobic conditions, has led us to believe that PG may play a larger role in soft rot development than previously thought (personal communication - Arthur Kelman).
CHAPTER III
MATERIALS AND METHODS

Culturing of Erwinia carotovora

All experiments were conducted using isolate EC14 of Erwinia carotovora (Jones) Holland, which was originally obtained from R. S. Dickey of Cornell University. EC14 was maintained on LB agar slants consisting of 1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar. Slants were inoculated and incubated at 30°C for 24 to 48 hours then stored at 4°C. Subculturing was performed every three to four weeks.

Bacteria were grown in a culture medium, 50 ml in 250 ml Erlenmeyer flasks containing 1% NaPP and Difco nutrient agar (8g/l). To some of the culture flasks 0.05% L-cysteine was added to serve as a reducing agent. Media was inoculated, by loop, from a slant and incubated overnight (8 to 12 hours) on a rotary shaker set at 150 revolutions per minute at room temperature (25 to 27°C). The overnight culture was used to inoculate 50 ml of the same medium in 250 ml culture flasks. A 10% inoculation rate was used.

Aerobic and Anaerobic Incubation of E. carotovora

Inoculated culture flasks were incubated either aerobically, free standing in an incubator, or anaerobically. Anaerobic conditions were obtained using a Gas-Pak anaerobic chamber and hydrogen and carbon dioxide generator envelopes (Beckton, Dickinson and Co. Cockeysville,
Md., 21030). The 0.5% palladium Gas-Pak catalyst pellets were changed before each experiment and a Gas-Pak aerobic indicator strip was placed in each chamber before it was sealed. Aerobic culture flasks and the anaerobic chamber which contained the culture flasks to be incubated anaerobically were placed side by side in the same incubator and incubated statically at either 24 or 30°C. Duplicate cultures were grown for each experiment. All cultures were incubated for 5 days. This was the time required, as determined previously in this laboratory, for wounded but uninoculated whole potato tubers to reach rot conditions when placed in the Gas-Pak anaerobic system (personal communication - Patrick D. Colyer). At the end of the incubation period bacterial populations of each culture were determined. A dilution series for each culture was performed in sterile distilled H₂O and 0.1 ml of each dilution was plated in triplicate, on Difco nutrient agar plates (23 g/1), which were supplemented with 0.4% casamino acids. Dilution plates were incubated at 30°C for 24 to 48 hours at which time the number of colonies was determined.

**Preparation of Crude Enzyme Samples**

After incubation bacterial cells were removed from the culture medium by centrifugation (12,000 g at 4°C for 20 min.) using a GSA rotor in a Sorvall RC2-B refrigerated centrifuge. The supernatant was retained and filtered through a Nalgene filter unit. Protein concentrations of the filtrate were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin was used
as a protein standard. The filtrates were then placed in 250 ml Erlenmeyer flasks and stored at 4°C until assayed for enzyme activity.

**Enzyme Assays**

Endopectate lyase (PL) activity was measured by the periodate-thiobarbituric acid assay (TBA) (47, 62). Endopolygalacturonase (PG) was determined using the Nelsons reducing group analysis (37). The ability of these enzymes to reduce the viscosity of a NaPP solution was determined using the viscometric assay (56).

**Preparation of substrates.** For the TBA assay and the viscometric assay for endoPL the substrate consisted of 1.4% NaPP in 0.05 M tris-HCl buffer (pH 8.3) containing, at a final concentration, 0.5 mMEDTA and 1.0 mM calcium chloride. For the Nelsons reducing group analysis and the viscometric assay of endoPG the substrate consisted of 1.4% NaPP in 0.05M PO₄ buffer (pH 6.0) containing, at a final concentration, 0.5 mM EDTA. The NaPP solutions for the assays were twice the final concentration and were used to make the above mentioned two substrates. In this way a constant concentration of NaPP could be maintained for each substrate prepared.

**Viscometric Assay.** In the viscometric assay 5.0 ml of substrate plus 1.0 ml of the crude enzyme preparation were added to an Ostwald-Fenske 300 viscometer, mixed and measured for depolymerization of NaPP over time. The readings for the viscometric assay were conducted at 30°C, which was maintained using a constant temperature water bath equipped
with a heating unit and a mixer. Relative activity was expressed as the reciprocal of the time in minutes for 50% loss in viscosity times $10^3$ (5, 6). Specific activity is the relative activity per milligram of protein per ml of enzyme solution. Autoclaved enzyme was used as a control.

TBA Assay. In this assay 100 ml of crude enzyme preparation was incubated with 100 ml of substrate at 30°C for the time required to reduce the viscosity of a NaPP solution by 50% as determined by the viscometric assay. An optical density of 0.3, measured at 548 nm in a Bausch and Lomb Spectronic 20 colorimeter, indicated the formation of 0.1 umoles of unsaturated product. A unit of activity was expressed as the amount of enzyme yielding an increase in absorbance of 0.1 in 1 hour, or the amount of enzyme yielding 1.0 umole of unsaturated product in one hour. Specific activity was expressed as units of activity per mg of protein per ml of enzyme solution.

Nelsons reducing group analysis. For determining the amount of reducing sugar released from NaPP, 100 ml of crude enzyme preparation was incubated with 250 ml of substrate at 30°C for the time required to reduce the viscosity of a NaPP solution by 50% as determined by the viscometric assay. A standard curve was prepared using D-galacturonic acid in concentrations ranging from 5 to 500 mg. Absorbance was measured at 500 nm in the Spectronic 20 colorimeter. Specific activity was expressed as the micromoles of product formed per mg of protein per ml of enzyme solution.
**In Vivo Pectic Enzyme Isolation**

An enzyme isolation was also performed from potato tissue which had been inoculated with *E. carotovora* isolate 14 and incubated anaerobically at 24 C.

**Preparation of Inoculum.** A culture of EC14 was grown in 1% NaPP and nutrient broth and incubated overnight on a rotary shaker set at 150 rpm at room temperature. The culture was then centrifuged (12,000g at 4 C for 20 min.) using a GSA rotor in a Sorval RC2-B refrigerated centrifuge. The supernatant was discarded and the bacterial cell pellet was retained and resuspended in 50 ml of sterile distilled H₂O and the bacterial population of this suspension was determined. A dilution series was performed in sterile distilled H₂O and 0.1 ml of the diluted cell suspension was plated, in triplicate, on nutrient Agar supplemented with 0.4% casamino acids. Dilution plates were incubated at 30 C for 24 hours, at which time the number of colonies was determined.

**Preparation and Assay of Crude Enzyme Samples.** The EC14 cell suspension was then used to inoculate potato pieces which had been surface sterilized in 10% Clorox for 30 sec., patted dry on paper towels and placed in beakers which had been sterilized. The
beakers were sealed in the Gas-Pak anaerobic chamber and the chamber was incubated at 24°C for 5 days at which time the rotted potato tissue and surrounding liquid were strained through several layers of cheesecloth, and centrifuged at 12,000 g at 4°C for 30 min. The supernatant was filtered through Nalgene filter units, placed in 250 ml Erlenmeyer flasks and stored at 4°C. Protein concentrations were determined and enzyme assays were conducted as previously described.

**Chemicals**

All the chemicals used in these experiments were obtained from the Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178, unless otherwise indicated. The sodium polypectate used in this study was that which was obtained from Sigma Chemical Co. prior to 1970.
CHAPTER IV
RESULTS

Maintenance of Anaerobic Culture Conditions

Cultures incubated anaerobically at 24°C maintained anaerobic conditions throughout the incubation period. Anaerobic conditions, for those cultures incubated anaerobically at 30°C, were slightly aerobic on the last two days of the incubation period for the second and third replicates of this culture treatment. A light-blue color on the indicator strip signified that complete anaerobic conditions had been lost, but that the level of \( \text{O}_2 \) present was still less than atmospheric. It is believed that the loss of anaerobic conditions in these cultures did not have a significant qualitative or quantitative effect on the enzymes produced. This is because: (1) the anaerobic conditions were lost so late in the incubation period; (2) the availability of \( \text{O}_2 \) for the bacterial cells incubated statically in the culture medium is probably not high; and (3) the bacterial population counts for the 30°C anaerobic cultures were not significantly different from those of the 24°C anaerobic cultures (Table 1).

Erwinia Carotovora Populations

The population of EC14 incubated at 24°C under aerobic conditions was higher than that of cells incubated under anaerobic conditions at 30°C (Table 1). Populations of bacteria anaerobically incubated were
TABLE 1

Means of bacterial populations of *Erwinia carotovora* isolate 14 culture incubated aerobically or anaerobically.\(^a\)

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Populations (cells/ml)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24°C Aerobic</td>
<td>1.41x10^8</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>1.40x10^8</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>2.95x10^4</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>6.86x10^3</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>3.72x10^7</td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>3.64x10^7</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>9.80x10^4</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>8.85x10^4</td>
</tr>
</tbody>
</table>

\(^a\)Means were based on three replicates for cultures grown in 1% NaPP Nutrient broth medium without L-cysteine and two replicates for those cultures grown in 1% NaPP Nutrient broth medium with 0.05% L-cysteine. Each replicate consisted of two duplicate cultures.

\(^b\)Bacterial populations were determined by plating dilutions on nutrient agar with 0.4% casamino acids. Plates were incubated at 30°C for 24 to 48 hours, after which time the number of colonies was determined.
markedly reduced when compared to the populations of aerobically incubated cultures. The 30°C anaerobic cultures had larger populations than those bacteria incubated under anaerobic conditions at 24°C. This may be due to the loss of anaerobic conditions which occurred late in the incubation period of the second and third replicates for this treatment. A reduction in bacterial populations was observed for cultures supplemented with 0.05% L-cysteine.

Because bacterial populations for anaerobic culture conditions were lower than the populations of aerobically cultured bacteria, the data is presented in two ways. In the first method data for extracellular protein concentrations and enzyme activity are presented unaltered; just as it was collected and calculated. The second method involved adjusting all extracellular protein concentrations and enzyme activities to the levels which would have been produced if that bacterial population had been $10^8$ cells/ml. This was performed in order to estimate the enzyme activities and the amount of protein produced had the population levels of the anaerobic cultures reached those of the aerobic cultures.

**Protein Concentrations**

The protein concentrations for each culture condition of EC14 were found to be fairly similar (Table 2). Cultures incubated at 24°C generally had slightly higher protein concentrations than cultures incubated at 30°C. The amount of protein produced by cultures supplemented with 0.05% L-cysteine was not markedly different from the protein concentrations of cultures grown without L-cysteine. When protein
TABLE 2

Extracellular protein concentrations for each culture of *Erwinia
carotovora* isolate 14 incubated aerobically or anaerobically.  

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Protein (mg/ml) in replicate b</th>
</tr>
</thead>
<tbody>
<tr>
<td>24°C Aerobic</td>
<td>0.135</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>0.142</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>0.135</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>0.138</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>0.10</td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>0.085</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>0.125</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>0.12</td>
</tr>
</tbody>
</table>

aBacterial cultures were grown in 1% NaPP Nutrient broth with and without L-cysteine supplementation.

bProtein concentrations were determined using the Bio-Rad Protein assay. Bovine serum albumin was used as a protein standard. Each replicate consisted of two duplicate cultures.
concentrations were adjusted to $10^8$ cells/ml a large increase in the protein concentrations of the anaerobically incubated cells resulted (Table 3). A slight increase was also noted for bacteria incubated under aerobic conditions at 30°C.

**Enzyme Activities**

*Viscometric Assay.* The ability of the crude extracellular enzyme preparations (endopectate lyase, PL; endopolygalacturonase, PG) to reduce the viscosity of a NaPP solution was measured using the viscometric assay (5, 6) (Table 4). The activities for the depolymerization of the acidic substrates were not dramatically affected by incubation at either 24 or 30°C. The activities remained fairly constant for both of these temperature treatments. The highest specific activities recorded for the depolymerization of the acidic substrate occurred for the 30 and 24°C aerobic cultures, with the 30°C culture being slightly higher. A slight decrease in the specific activity values of the viscometric assay was observed for enzyme solutions obtained from cultures which had contained 0.05% L-cysteine. When all activity values for the viscometric assays of the various culture conditions were adjusted to $10^8$ cells/ml a large increase was observed for the 24 and 30°C anaerobic cultures, when compared to the aerobic cultures (Table 5).

A marked difference in the specific activities for the depolymerization of the alkaline substrate was observed for the two temperatures tested (Table 4). All activity values, for the crude enzyme solution prepared from 30°C cultures, were higher than those
TABLE 3

Extracellular protein concentrations for each culture of Erwinia carotovora isolate 14 incubated aerobically or anaerobically and adjusted to $10^8$ cells/ml.a

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Protein (mg/ml) in replicate;(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>24°C Aerobic</td>
<td>0.095</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>0.101</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>$4.57\times10^2$</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>$2.02\times10^3$</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>0.269</td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>0.234</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>$1.27\times10^2$</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>$1.36\times10^2$</td>
</tr>
</tbody>
</table>

\(^a\)Bacterial cultures were grown in 1% NaPP nutrient broth with and without L-cysteine supplementation.

\(^b\)Protein concentration were determined using the Bio-Rad Protein assay and values were adjusted to $10^8$ cells/ml. Bovine serum albumin was used as a protein standard. Each replicate consisted of two duplicate cultures.
## TABLE 4

Means of viscometric assay values of crude extracellular enzyme preparations of *Erwinia carotovora* isolate 14 incubated aerobically or anaerobically.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Relative Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Specific Activity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.0</td>
<td>pH 8.3</td>
</tr>
<tr>
<td>24°C Aerobic</td>
<td>7.91</td>
<td>12.2</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>5.81</td>
<td>9.98</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>5.75</td>
<td>12.5</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>5.64</td>
<td>9.34</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>7.60</td>
<td>24.25</td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>5.23</td>
<td>18.06</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>5.97</td>
<td>18.72</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>5.30</td>
<td>13.23</td>
</tr>
</tbody>
</table>

<sup>a</sup>The alkaline substrate consisted of 1.4% NaPP in 0.05 M Tris-HCl buffer at pH 8.3 plus 0.5 mM EDTA and 1.0 mM calcium for measuring the pectate lyase activity. The acidic substrate contained 1.4% NaPP in 0.05 M PO<sub>4</sub> buffer at pH 6.0 plus 0.5 mM EDTA for measuring polygalacturonicate activity. Five ml of substrate and 1.0 ml of crude enzyme solution were added to Ostwald-Fenske 300 viscometers, mixed and the depolymerization of NaPP was measured over time. Means were based on the first two replicates of these experiments, each replicate consisted of two duplicate cultures.

<sup>b</sup>Relative activity is the reciprocal of the time in minutes to reach 50% reduction in viscosity times 10³(5, 6).

<sup>c</sup>Specific activity is the relative activity per milligram of protein per ml of enzyme solution.
TABLE 5

Means of viscometric assay values of crude extracellular enzyme preparations of *Erwinia carotovora* isolate 14 incubated aerobically or anaerobically and adjusted to $10^8$ cells/ml.a

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Enzyme Activity</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative Activity$^b$</td>
<td>Specific Activity$^c$</td>
</tr>
<tr>
<td></td>
<td>pH 6.0</td>
<td>pH 8.3</td>
</tr>
<tr>
<td>24°C Aerobic</td>
<td>5.6</td>
<td>8.67</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>4.15</td>
<td>7.13</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>$1.95 \times 10^4$</td>
<td>$4.24 \times 10^4$</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>$8.22 \times 10^4$</td>
<td>$1.36 \times 10^5$</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>20.43</td>
<td>65.19</td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>14.36</td>
<td>49.68</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>$6.09 \times 10^3$</td>
<td>$1.91 \times 10^4$</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>$5.99 \times 10^3$</td>
<td>$1.49 \times 10^4$</td>
</tr>
</tbody>
</table>

$a$The alkaline substrate consisted of 1.4% NaPP in 0.05 M Tris-HCl buffer at pH 8.3 plus 0.5 mM EDTA and 1.0 mM calcium for measuring the pectate lyase activity. The acidic substrate contained 1.4% NaPP in 0.05 M PO$_4$ buffer at pH 6.0 plus 0.5 mM EDTA for measuring polygalacturonate activity. Five ml of substrate and 1.0 ml of crude enzyme solution were added to Otswald-Fenske 300 viscometers, mixed, and the depolymerization of NaPP was measured over time. Means were based on the first two replicates of these experiments, each replicate consisted of two duplicate cultures.

$^b$Relative activity is the reciprocal of the time in minutes to reach 50% reduction in viscosity times $10^3$ per $10^8$ cells/ml (5, 6).

$^c$Specific activity is the relative activity per milligram of protein per ml of the enzyme solution per $10^8$ cells/ml.
values obtained for the 24°C cultures. This increase was so high that even the enzyme solution prepared from 30°C anaerobic culture approached the values for 24°C aerobic cultures. All aerobic cultures had higher activity than their anaerobic counterparts. Cultures which were supplemented with 0.05% L-cysteine had decreased specific activity values. However, the specific activity values of anaerobic cultures which had been adjusted to $10^8$ cells/ml were 100 to 10,000 x greater than those for the aerobic cultures at 24°C and 100 x greater than those for the aerobic cultures at 30°C (Table 5).

**TBA Assay.** The PL specific activity values, as measured by the thio-barbituric acid (TBA) assay (47, 62) (Table 6) agreed with the results obtained in the viscometric assay (Table 4). All PL specific activity values obtained from crude enzyme solutions of 30°C cultures were higher than those values obtained from 24°C cultures. Unlike the viscometric assay, this also included the 24°C aerobic cultures. The PL activity values were higher for cultures incubated under aerobic conditions than for cultures incubated anaerobically. Also, cultures which were not supplemented with L-cysteine had higher activity values than cultures which were supplemented with 0.05% L-cysteine. When PL activity values were adjusted to $10^8$ cells/ml an appreciable increase was obtained for anaerobically incubated cultures (Table 7). The PL activities of cultures incubated under anaerobic conditions at 24 and 30°C were about 100 x greater than the activity values obtained for cultures incubated under aerobic conditions at 24 and 30°C. Cultures incubated anaerobically
TABLE 6

Means of pectate lyase (PL) activities of crude extracellular enzyme preparations of *Erwinia carotovora* isolate 14 incubated aerobically or anaerobically.a

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>PL Activity^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>u moles Product^c</td>
</tr>
<tr>
<td>24°C Aerobic</td>
<td>1.22</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>0.744</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>0.940</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>0.547</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>5.007</td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>2.771</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>2.792</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>1.665</td>
</tr>
</tbody>
</table>

^a Means were based on the first two replicates of these experiments, each replicate consisted of two duplicate cultures.

^b Reaction mixtures contained 100 ul of crude enzyme solution plus 100 ul of 1.4% NaPP in 0.05mM Tris-HCl buffer pH 8.3 and 0.5 mM EDTA with 1.0 mM calcium and analyzed by the TBA assay (47, 62).

^c Expressed as the umoles of unsaturated product formed per ml of enzyme solution per hour.

^d Expressed as the umoles of product formed per ml of enzyme solution per hour per milligram of protein per ml.
TABLE 7

Means of calculated pectate lyase (PL) activities of crude extracellular enzyme preparation of *Erwinia carotovora* isolate 14 incubated aerobically or anaerobically and adjusted to 10^8 cells/ml.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>u moles Product^c</th>
<th>Specific Activity^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>24°C Aerobic</td>
<td>0.867</td>
<td>7.50</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>0.531</td>
<td>3.90</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>3.19x10^3</td>
<td>2.42x10^4</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>7.97x10^3</td>
<td>6.03x10^4</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>13.46</td>
<td>1.31x10^2</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>7.62</td>
<td>7.86x10^1</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>2.85x10^3</td>
<td>2.40x10^4</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>1.88x10^3</td>
<td>1.78x10^4</td>
</tr>
</tbody>
</table>

^aMeans were based on the first two replicates of these experiments; each replicate consisted of two duplicate cultures.

^bReaction mixtures contained 100 ul of crude enzyme solution plus 100 ul of 1.4% NaPP in 0.05 mM Tris-HCl buffer pH 8.3 and 0.5 mM EDTA with 1.0 nM calcium and analyzed by the TBA assay (47, 62).

^cExpressed as the umoles of unsaturated product formed per ml of enzyme solution per hour per 10^8 cells/ml.

^dExpressed as the umoles of product formed per ml of enzyme solution per hour per milligram of protein per ml per 10^8 cells/ml.
at 24°C had slightly higher activity values than those incubated under anaerobic conditions at 30°C.

Nelson reducing group analysis. The specific activity of PG in crude extracellular enzyme solutions was measured using the Nelsons reducing group analysis (37) (Table 8). These values agreed with the activity values obtained, for the same enzyme solutions, in the viscometric assay (Table 4). The umoles of reducing product formed by the 24°C incubated cultures were higher than the amounts of product formed in the corresponding cultures incubated at 30°C. However, the specific activity values obtained for 30°C cultures were higher than those of the 24°C cultures. The 24 and 30°C aerobic cultures possessed specific activity values which were twice as high as those of anaerobically incubated cultures at the same temperature. Once again cultures which were supplemented with 0.05% L-cysteine had reduced specific activity values when compared to cultures without L-cysteine. When data for PG specific activity were adjusted to 10^8 cells/ml the activity values of cultures incubated under anaerobic conditions increased by about 1000-fold for 24°C cultures and about 10-fold for 30°C cultures, when compared to cultures incubated under aerobic conditions at these same temperatures (Table 9). Cultures incubated anaerobically at 24°C had slightly higher specific activity values than anaerobically incubated cultures at 30°C.

In Vivo Enzyme Isolation

A 1.08x10^9 cells/ml suspension of E. carotovora isolate 14, in
TABLE 8

Means of polygalacturonase (PG) activities of crude extracellular enzyme preparation of *Erwinia carotovora* isolate 14 incubated aerobically or anaerobically\(^a\)

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>PG Activity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles Product(^c)</td>
</tr>
<tr>
<td>24°C Aerobic</td>
<td>9.895</td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>5.46</td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>5.77</td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>5.28</td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>9.35</td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>4.56</td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>5.305</td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>4.79</td>
</tr>
</tbody>
</table>

\(^a\)Means were based on the first two replicates of these experiments, each replicate consisted of two duplicate cultures.

\(^b\)Reaction mixtures contained 100 μl of crude enzyme solution plus 250 ml of 1.4% NaPP in 0.05 M PO\(_4\) buffer pH 6.0 and 0.5 mM EDTA and assayed by the Nelsons reducing group analysis (37).

\(^c\)Expressed as the umoles of free reducing sugar released from NaPP per ml of enzyme solution per hour.

\(^d\)Expressed as the umoles of free reducing sugar released per milligram of protein per ml of enzyme solution.
TABLE 9

Means of calculated polygalacturonase (PG) activities of crude extracellular enzyme preparation of Erwinia carotovora isolate 14 incubated aerobically and anaerobically and adjusted to $10^8$ cells/ml.\(^a\)

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>PG Activity(^b)</th>
<th>(\mu) moles Product(^c)</th>
<th>Specific Activity(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24°C Aerobic</td>
<td>7.03</td>
<td>62.00</td>
<td></td>
</tr>
<tr>
<td>24°C Aerobic with L-cys.</td>
<td>3.90</td>
<td>28.54</td>
<td></td>
</tr>
<tr>
<td>24°C Anaerobic</td>
<td>1.96x10^4</td>
<td>1.50x10^5</td>
<td></td>
</tr>
<tr>
<td>24°C Anaerobic with L-cys.</td>
<td>7.69x10^4</td>
<td>5.81x10^5</td>
<td></td>
</tr>
<tr>
<td>30°C Aerobic</td>
<td>25.13</td>
<td>238.87</td>
<td></td>
</tr>
<tr>
<td>30°C Aerobic with L-cys.</td>
<td>12.54</td>
<td>125.58</td>
<td></td>
</tr>
<tr>
<td>30°C Anaerobic</td>
<td>5.41x10^3</td>
<td>4.61x10^4</td>
<td></td>
</tr>
<tr>
<td>30°C Anaerobic with L-cys.</td>
<td>5.41x10^3</td>
<td>5.13x10^4</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Means were based on the first two replicates of these experiments; each replicate consisted of two duplicate cultures.

\(^b\)Reaction mixtures contained 100 ul of crude enzyme solution plus 250 ul of 1.4% NaPP in 0.05 M PO\(_4\) buffer pH 6.0 and 0.5 mM EDTA and were assayed by the Nelson reducing group analysis (37).

\(^c\)Expressed as the \(\mu\)moles of free reducing sugar released from NaPP per ml of enzyme solution per hour per \(10^8\) cells/ml.

\(^d\)Expressed as the \(\mu\)moles of free reducing sugar released per milligram of protein per ml of enzyme solution.
sterile distilled H$_2$O, was used to inoculate potato tuber tissue slices. These slices were then incubated under anaerobic conditions at 24 C. When the incubation period was completed a crude extracellular enzyme solution was prepared as previously described.

The protein contraction of the crude enzyme solution was determined to be 0.735 mg/ml. This solution was found to reduce the viscosity of a 1.4% NaPP solution (pH 8.3) by 50%, in 3.6 minutes. The enzyme activity of this crude enzyme solution was 373.25 (Table 10). The umoles of unsaturated products, as measured by the TBA assay (47, 62) were 16.67 (Table 10).

The viscosity of a 1.4% NaPP solution (pH 6.0) was reduced to 50%, by the above mentioned extracellular enzyme solution, in 28 minutes, with a corresponding activity of 47.91 (Table 10). The amount of free reducing groups released from NaPP, as measured by the Nelsons reducing group analysis (37), was 19.11 umoles (Table 10).
### TABLE 10

Activities of pectate lyase and polygalacturonase isolated from macerated potato tuber tissue inoculated with *Erwinia carotovora* isolate 14 and incubated anaerobically at 24°C

<table>
<thead>
<tr>
<th>Assay Performed</th>
<th>Enzyme Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH 6.0</th>
<th>pH 8.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBA Assay&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>-</td>
<td>16.67</td>
</tr>
<tr>
<td>Nelsons Reducing Sugar Analysis&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.11</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Viscometric Assay&lt;sup&gt;d&lt;/sup&gt;</td>
<td>47.91</td>
<td>373.25</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The alkaline substrate utilized in enzyme activity assays consisted of 1.4% NaPP in 0.05 M Tris-HCl buffer at pH 8.3 plus 0.5 mM EDTA and 1.0 mM calcium. The acidic substrate utilized in enzyme activity assays contained 1.4% NaPP in 0.05 M PO₄ buffer at pH 6.0 plus 0.5 mM EDTA.

<sup>b</sup>Reaction mixture for the thiobarbituric acid assay (47, 62) contained 100 ul of crude extracellular enzyme solution plus 100 ul of the alkaline substrate. Enzyme activity is expressed as the umoles of unsaturated product formed per ml of enzyme solution per hour.

<sup>c</sup>Reaction mixture for the Nelsons reducing sugar analysis (37) contained 100 ul of crude extracellular enzyme solution plus 250 ul of the acidic substrate. Enzyme activity is expressed as the umoles free reducing sugar released from NaPP per ml of enzyme solution per hour.

<sup>d</sup>In the viscometric assay 5.0 ml of substrate and 1.0 ml of crude extracellular enzyme solution were added to Ostwald-Fenske 300 viscometers, mixed, and the depolymerization of NaPP was measured over time. Enzyme activity is expressed as the reciprocal of the time in minutes to reach 50% reduction in viscosity times 10³ per milligram of protein per ml of enzyme solution (5, 6).
CHAPTER V
DISCUSSION

Environmental conditions and the types and numbers of microorganisms in or on the potato tuber have been found to be major determinants in the development of soft rot of potato tubers (10, 15, 16, 25, 26, 27, 38, 44, 45, 50). The question arises as to whether the increase in the development of soft rot occurs as a result of a reduction in the resistance response of the tuber during anaerobic conditions or an increase in the metabolism of pectic enzyme synthesis in the pathogen under anaerobic conditions. This investigation sought to determine if the production of the pectic enzymes necessary for soft rot development were affected by either temperature or more importantly the availability of oxygen.

To date most investigators have believed that extracellular PL is the primary cause of tissue maceration and cellular death in potato tubers rotted by EC 14 (33, 57). However, the observation that the rotted tuber tends to have a lower pH than normal (near pH 6.0 - the optimal pH for PG activity) and recent studies in controlled environmental chambers, with whole potato tubers under anaerobic conditions, has led us to believe that extracellular PG may play a larger role in soft rot development than previously thought.

A reduction in the population of EC 14 occurred when this bacterium was incubated under anaerobic conditions. Wells (63) and Menely and Stanghellini (29) reported that the growth rate, in vitro of Erwinia
spp. was reduced when cells were grown in low oxygen atmospheres (HO 3%). In light of these data a reduction in the population of anaerobically incubated EC 14 cultures is not surprising. What is surprising, however, is the observation that extracellular protein concentrations for EC 14 cultures incubated aerobically and anaerobically were approximately the same.

To determine the importance of PL or PG in the development of soft rot in potato tubers the levels of production of these enzymes were studied under a variety of culture conditions. Our results indicate that extracellular PL levels from EC 14 were higher than that of the PG levels produced from EC 14. Perombelon and Ghanaker (43) determined that in seven of nine strains of *E. carotovora* var. *carotovora* the production of PL was as great at 15°C as at 30°C. The remaining two strains, however, produced less PL at 15°C than at 30°C. The synthesis of PL, for our strain of *E. carotovora* was shown to be dependent on temperature, more than on any other environmental parameter. Higher levels of PL activity were found for cultures incubated either aerobically or anaerobically at 30°C compared to those cultures incubated at 24°C. These results indicate that our strain of EC 14 may be similar to the two strains described above. Also, this laboratory has always incubated the EC 14 strain of *E. carotovora* at 30°C and the regulatory mechanism(s) involved in the production of extracellular PL levels may have become selective for this temperature.

Endo PG production was dependent on the availability of oxygen. PG activities were higher for cultures incubated aerobically at 24 and
30 C than for those cultures incubated anaerobically at the same temperatures. Because PL activities were reduced at 24 C the levels of PG, which remained constant for aerobically incubated cultures, becomes more important in the maceration of the pectic portion of the cell wall. It is believed, however, that the low pH values observed in soft-rotted potato tuber tissue cannot be attributed to PG levels alone. This pH reduction may be the result of various organic acids which are liberated from cell walls which have been degraded by soft rot organisms.

When pectic enzymes were isolated from anaerobically treated, macerated potato tuber tissue, 0.735 mg/ml of protein was detected in the crude extracellular enzyme solution. The viscometric assay results indicated, once again, that PL was produced in higher amounts and was more effective in reducing the viscosity of a NaPP solution, than was PG. PL from the in vivo enzyme preparation was able to cause a reduction in viscosity of 50% 20 times faster than any crude enzyme solution prepared from in vitro cultures. While PG did not reduce the viscosity of a NaPP solution as rapidly as PL, a 50% reduction in viscosity did occur at a rate 4.5 times faster than that obtained for in vitro enzyme solutions.

When all activity values were adjusted to the levels which would have been obtained by a $10^8$ cells/ml culture, a marked increase in the activity values obtained for all anaerobically incubated cultures resulted. It was also observed that more protein was produced, on a per cell basis, in anaerobic than in aerobic cultures. This raises the question of what mechanism(s) is involved in controlling these phenomena. Two
possibilities are evident: either the bacteria are transporting existing intracellular proteins out of the cell more efficiently or the synthesis of all extracellular proteins by the cell are greatly increased. A combination of these two possibilities may also take place. In this scenario, bacteria which are placed under anaerobic conditions determine that they are in a stressful situation and are capable of utilizing one, or both, of these mechanisms to insure a greater chance of survival.

The second of these two mechanisms involves an acceleration of the bacterial metabolisms once it is placed under stress conditions. This would increase the amount of pectic enzymes each bacterium produces, and enable a smaller number of cells to initiate soft-rot symptoms. Anaerobic conditions may also increase the amount on nonpectolytic enzymes synthesized. Several researchers have reported that extracellular enzymes such as protease, phosphotidase, galactanase, mannase, galactosidase and xylanase may also be involved in the pathogenesis of soft rot conditions (21, 56, 57, 60).

Intracellular endo PL enzymes were characterized from nonpathogenic klebsiella oxytoca and yersinia enterocolitica (4). The klebsiella and yersinia PLs were shown to have significant physical and chemical differences when compared to a PL obtained from a pathogenic strain of E. chrysanthemi. These enzymes were not transported out of the cell and did not macerate plant tissue unless pectin esterase (PE) was endogenously supplied. When placed under anaerobic conditions enzymes such as these, or the intracellular pectin depolymerase complex described
by Stack et al. (52), may be transported out of the cell and aid the bacterium in obtaining nutrients. The mechanism by which certain enzymes, and not others, are transported out of the cell is not clearly understood, but a recognition response at the membrane level may be involved. Recognition might be regulated by the ionic charges or antigenic properties of the enzyme.

When assessing the importance of endo PG or PL, in pathogenesis, it is crucial to keep in mind the effects of the end products of both of these enzymes. Collmer (13, 14) showed that unsaturated and saturated di-galacturonic acid (4DG and DG) can be further degraded by oligogalacturonate lyase (OGL), which was not assayed in this study, into 4-deoxy-5 keturonic acid (DK). DK, or its precursor, was found to be an effective inducer of PL synthesis in E. chrysanthemi. Stack (51, 52) found that, at pH 6.0, the cleavage of NaPP by PDIa (endo PG) resulted in the production of saturated products only. Traces of saturated digalacturonic acid were produced and may prove (not tested with EC 14) to be an effective inducer of PL. If a system of inducer formation, similar to that found for E. chrysanthemi, is observed for E. carotovora, the importance of PG activity in soft rot development will be greatly increased.

In order to more fully understand the occurrence and prevention of soft rot of potato tuber further research is required. New areas of investigation will include studying the role of the intracellular pectic enzyme complex, produced by EC 14, in determining PL synthesis. Stack (51, 52) determined that two of the enzymes identified in the intracellular
enzyme complex of EC 14, PDII and III, exhibited exo modes of action and were able to produce unsaturated digalacturonic acid which has been shown to be an effective inducer of PL synthesis in *E. carotovora*. The effects the environmental conditions necessary for soft rot development, have on these regulatory mechanisms should be a focus of further research. By obtaining this information post-harvest physiologists and disease physiologist can cooperate in determining the storage methods which will maximize post harvest life and minimize the losses which occur as a result of soft rot diseases.


