

# Pectinolytic and Pectolytic Microorganisms

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## 18.1 INTRODUCTION

Pectic substances are important cell wall components of higher plants, particularly dicots. The specific functions that pectic materials perform in the cell wall are not understood. However, it appears that they are important in cementing plant cells together. BeMiller<sup>6</sup> has reviewed pectin structure. Pectic substances are polymers of D-galacturonic acid residues glycosidically linked alpha-1,4. Pectin molecules contain occasional rhamnose units with 1,2 linkages in the main chain. They also have side chains on both the galacturonic acid and rhamnose residues that contain mainly galactose and arabinose residues. The carboxyl groups of galacturonic acid residues are usually methylesterified to a substantial degree. This has major effects on physical properties of pectin, such as gelation. The degree of methylation in plants has been found to range from about 40% to 90% of the carboxyl groups.<sup>2</sup> Data on the distribution of carboxyl groups are limited, but it appears most often to be random in pectin isolated to minimize enzymatic or chemical modification.<sup>2</sup> In some plants, substantial numbers of the hydroxyl groups of galacturonic acid residues are acetylated. This modification inhibits pectin gelation.<sup>6</sup>

The nomenclature for pectic substances has been somewhat variable and confused over the years. For the purpose of this chapter, pectic substances is an inclusive term for galacturonic acid-containing polymers from plant cell walls. Pectin is used for pectic substances with a substantial fraction of the galacturonic acid carboxyl groups esterified. Pectic acid refers to polymers with a negligible amount of the carboxyl groups esterified. Polypectate or pectate refers to pectic acid with carboxyl groups in the salt form. Pectinolytic refers to the degradation of pectin and pectolytic to the degradation of pectic acid or pectate. Commercially available pectin of the type used for microbiological or enzymatic assays generally has >60% methylation. Pectic acid or polypectate is <5% esterified.

### 18.11 Sources of Pectinolytic and Pectolytic Enzymes

Most pectin-degrading organisms are associated with raw agricultural products and with soil. Up to 10% of the organisms in soil have been shown to be pectinolytic.<sup>23</sup> These include, but are not limited to, bacteria in the genera *Achromobacter*, *Aeromonas*,

*Arthrobacter*, *Agrobacterium*, *Enterobacter*, *Bacillus*, *Clostridium*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, and *Xanthomonas*,<sup>54,59</sup> as well as in yeasts, molds, protozoa, and nematodes.<sup>3</sup> Many of these organisms are plant pathogens.<sup>3,17</sup> A survey of yeasts from 13 genera<sup>8</sup> found pectinolytic activity produced by 69 of 207 strains tested. Pectolytic activity is not common in lactic acid bacteria. However, it has now been reported to occur in at least one strain of *Leuconostoc mesenteroides*,<sup>30</sup> *Lactobacillus acidophilus*,<sup>34</sup> *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactococcus lactis*.<sup>31</sup> The molecular biology of pectolytic enzymes from plant pathogenic erwiniae has been extensively investigated.<sup>12,36,51</sup> The discussion that follows refers to aerobic procedures. However, the detection of anaerobic pectinolytic bacteria also has been described.<sup>41,46</sup>

## 18.2 DETECTING PECTINOLYTIC AND PECTOLYTIC ORGANISMS

The basic method used to detect pectinolytic or pectolytic organisms has been to grow the organisms on a gel medium that contains pectin or pectate substrates, respectively. Production of enzymes by a culture is detected either by observing depressions in the gel around the colony where the substrate has been degraded or by flooding the plate with a precipitant solution. Around producer colonies a clear zone will appear where the substrate has degraded to the point that precipitation does not occur, while non-producing colonies will be surrounded by opaque gel containing the non-degraded pectin or pectate substrate. Wieringa<sup>60</sup> reported the first medium of this type. Over the years, many variations of this theme have been developed to address particular problems of sample handling, enzyme specificity, sensitivity, or isolation of organisms from the plates. For example, a researcher can cut holes in agar gel plates with a cork borer in order to assay liquid samples, such as culture filtrates, for enzyme activity. This is the so-called well plate or cup plate technique for enzyme assays.

### 18.21 Pectate and Pectin Lyase Producers

Considerable research has been conducted on techniques to detect pectate lyase-producing organisms. This is because these enzymes from *Erwinia* species have been cloned into *Escherichia coli*. Since lyases have alkaline pH optima, while poly-galacturonases have acidic optima, a medium pH of 7.0 or above is the main

parameter used to distinguish pectate or pectin lyase producers from polygalacturonase producers. Durrands and Cooper<sup>18</sup> provide an example of this approach, in which media were designed to detect polygalacturonase and pectin lyase production by *Verticillium albo-atrum* mutants. Roberts et al.<sup>52,53</sup> and Allen et al.<sup>1</sup> used the pH 8.5 PEC-YA medium of Starr et al.<sup>57</sup> to clone pectate lyase genes from *Erwinia carotovora*.

### 18.211 Bacterial Pectate Lyases

Several media have been developed for the detection of bacteria that produce pectate lyases. King and Vaughn<sup>35</sup> developed a pectate medium with a pH of 7.0 that contained crystal violet to make it selective for gram-negative bacteria. Adding cycloheximide further inhibits the growth of yeasts and molds. Detection of pectolytic colonies is based on formation of depressions in the pectate gel because of enzymatic degradation.

Hankin et al.<sup>24</sup> used a mineral medium with 0.1% yeast extract, pectin, and agar to detect pectolytic colonies of *Erwinia* and *Pseudomonas*. A 1% aqueous solution of hexadecyltrimethylammonium bromide<sup>29</sup> was used to precipitate non-degraded substrate so that pectolytic colonies showed a clear zone on a white background. The researchers emphasized that a high phosphate level in the medium was needed to observe pectolytic activity. Sands et al.<sup>55</sup> modified the medium of King and Vaughn<sup>35</sup> by using 2% pectin and 1.5% agar instead of 7% polypectate. They then added a mixture of novobiocin, penicillin G, and cycloheximide to make the medium selective for fluorescent pseudomonads. Hexadecyltrimethylammonium bromide solution was used to precipitate the pectin for visualization of clear zones around pectolytic colonies.

Cuppels and Kelman<sup>15</sup> did a detailed evaluation of the selectivity for and recovery of *Erwinia* from natural samples using another pectate medium containing crystal violet to prevent the growth of unwanted organisms. The medium gave an excellent recovery of pectolytic *Erwinia*, but did allow growth and enzyme production by some pseudomonads. This medium has also been used to isolate pectolytic strains of *Cytophaga johnsonae* from spoiled, fresh bell peppers and watermelon.<sup>39</sup> Woodward and Robinson<sup>62</sup> modified crystal violet medium by addition of proteose peptone to reduce the time required to detect pectolytic *Erwinia* and improve pit formation. Pierce and McCain<sup>48</sup> modified the medium of Miller and Schroth<sup>43</sup> to selectively plate for pectolytic *Erwinia*. They found it to be selective for *Erwinia* and to improve recovery compared to the Cuppels and Kelman crystal violet medium. With all of these media, it has been reported that not all commercial polypectate preparations give suitable gelation. A procedure has been described to produce polypectate from orange peel and apple pulp that will give a good gel.<sup>13</sup>

An essential element of work to identify and characterize pectin-degrading genes from *Erwinia chrysanthemi* was the development of plating techniques to make it possible to identify clones that contained the genes of interest. Keen et al.<sup>33</sup> described the isolation of *E. coli* clones that contain pectate lyase genes. They used a pectate agar at pH 8.0. After incubating samples, they detected lyase activity by flooding the plates with 1 M CaCl<sub>2</sub>. A white halo formed around positive clones. Kotoujansky et al.<sup>37</sup> developed a technique to isolate clones of lambda-L47-1 phage to which pectate lyase genes had been transferred. They used a medium for *E. coli*, the phage host, in one layer, and a pectate medium in the bottom layer. The two gels were separated by a nylon membrane that allowed enzymes to diffuse into the pectate

layer, but prevented transfer of the phage. Zones with pectate lyase activity were visualized by removing the nylon membrane and the upper gel and flooding the pectate medium with 1 M CaCl<sub>2</sub>. The nylon membrane was then placed back on the plate so that phage clones could be isolated from the appropriate plaque.

### 18.212 Bacterial Pectin Lyases

Pectin lyases should give clearing zones on plates with pectin as the substrate. However, plates are not very sensitive for this group of pectic enzymes, and the zones produced can be indistinct.<sup>56</sup> Detection is accomplished by spectrophotometric assays at 235 nm on culture filtrate samples with high methoxyl pectin as substrate. Pectin lyases will give little or no measurable activity with polypectate as the substrate.

### 18.213 Fungal Pectate Lyases

Hankin and Anagnostakis<sup>22</sup> describe a plate technique with a medium that contains 1% pectin, 0.2% yeast extract, mineral salts, and 3% agar adjusted to pH 7.0. After a 3- to 5-day incubation period, plates are flooded with 1% aqueous hexadecyltrimethylammonium bromide to precipitate non-degraded pectin. Clear zones occur around colonies that produce pectate lyase. If the precipitant is not allowed to remain in contact with fungal cells for more than 5 min, viable colonies can be isolated from the flooded plates. For fungal samples from natural isolations that contain bacteria, a mixture of the antibiotics neomycin and chloramphenicol provides control of bacterial growth with the least inhibition of growth or enzyme production by fungi. However, the authors emphasize that fungi should be purified and enzyme production checked in the absence of the antibiotics.

## 18.22 Fungal Polygalacturonase Producers

The detection of polygalacturonases by plate assays has generally been done simply by lowering the pH of a medium designed for detection of pectate lyase to 6 or below, so that polygalacturonases will be active and pectate lyases will be inactive.<sup>22,58</sup>

### 18.23 Detecting Pectic Enzymes during Germination of Fungal Spores

Hagerman et al.<sup>21</sup> described a plate procedure for detection of pectolytic enzymes, protease, and cellulase activity during germination of *Botrytis cinerea* spores. The method is very sensitive for the detection of lyases and pectinesterase, but it is considerably less sensitive for polygalacturonase.

### 18.24 Evaluating Macerating Activity of Pectic Enzymes

Mussell and Morre<sup>44</sup> analyzed in detail factors affecting the maceration of cucumber tissue by commercial polygalacturonase from *Aspergillus niger*, basing their procedure on the measurement of weight loss of the cucumber tissue after enzyme treatment. They pointed out that tissue maceration assay for polygalacturonase activity was about 500 times more sensitive than viscosity assays. Ishii<sup>28</sup> developed a procedure to evaluate the maceration of potato, onion, and radish tissues by measuring the volume of separated cells released from tissue samples. A polygalacturonase and pectate lyase, separately and in combination with *Aspergillus japonicus*, were used. Both enzymes caused tissue maceration, but the relative activity of the enzymes varied with the plant tissue.

