PECTINOLYTIC AND PECTOLYTIC MICROORGANISMS

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14.1 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[6] 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 methyl-esterified 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.[2] 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.[2] In some plants, substantial numbers of the hydroxyl groups of galacturonic acid residues are acetylated. This modification inhibits pectin gelation.[6]

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.

14.11   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.[22] These include, but are not limited to, bacteria in the genera *Achromobacter, Aeromonas, Arthrobacter, Agrobacterium, Enterobacter, Bacillus, Clostridium, Erwinia, Flavobacterium, Pseudomonas, Xanthomonas*,[48, 53] and many yeasts, molds, protozoa, and nematodes.[3] Many of these organisms are plant pathogens.[3, 16] Recently, pectolytic activity was found in a strain of *Leuconostoc mesenteroides*.[29] This is the first report of pectolytic activity in lactic acid bacteria. Studies have reviewed the role of pectic enzymes, their regulation, and their molecular genetics in plant pathogenesis by plant pathogenic erwiniae.[11, 12, 33] The discussion that follows refers to aerobic procedures. However, the detection of anaerobic pectinolytic bacteria also has been described.[38, 43]

14.2   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 nonproducing colonies will be surrounded by opaque gel containing the nondegraded pectin or pectate substrate. Wieringa[54] 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.

14.21   Pectate and Pectin Lyase Producers

Since publication of the previous edition of this book, 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 polygalact-

uronases 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[17] provided a recent sample of this approach, in which media were designed to detect polygalacturonase and pectin lyase production by *Verticillium albo-atrum* mutants. Roberts et al.[46, 47] and Allen et al.[1] used the pH 8.5 PEC-YA medium of Starr et al.[51] to clone pectate lyase genes from *Erwinia carotovora*.

14.211   Bacterial pectate lyases

Several media have been developed for the detection of bacteria that produce pectate lyases. King and Vaughn[32] 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.[23] 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[28] was used to precipitate nondegraded 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.[49] modified the medium of King and Vaughn[32] 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[15] 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.[36] It has been shown that not all commercial polypectate samples will give a suitable gel in this medium, and a procedure has been described to produce polypectate from orange peel and apple pulp that will give a good gel.[13] Starr et al.[51] used a similar medium without crystal violet to demonstrate pectolytic activity in *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Klebsiella pneumoniae*.

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.[30] 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 CaCl₂. A white halo formed around positive clones. Kotoujansky et al.[34] 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 CaCl₂. The nylon membrane was then placed back on the plate so that phage clones could be isolated from the appropriate plaque.

14.212   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.[50] Detection is accomplished by spectrophotometric assays at 235 nm on culture filtrate samples with high methyoxl pectin as substrate. Pectin lyases will give little or no measurable activity with polypectate as the substrate.

14.213   Fungal pectate lyases

Hankin and Anagnostakis[21] 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 nondegraded 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.

14.22   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.[21, 52]

14.23   Detecting Pectic Enzymes During Germination of Fungal Spores

Hagerman et al.[20] 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 pectin-esterase, but it is considerably less sensitive for polygalacturonase.

14.24   Evaluating Macerating Activity of Pectic Enzymes

Mussell and Morre[41] 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[27] 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.

14.3   VISUALIZATION AND ASSAY OF PECTIC ENZYMES

14.31   Detecting Pectic Enzymes in Electrophoresis Gels

The visualization of pectic enzymes in gels following electrophoresis has been described. Cruickshank and Wade[14] incorporated citrus pectin into acrylamide slab gels. By using suitable incubation and staining procedures, they were able to differentiate pectate lyases, polygalacturonases, and pectin-esterases in the gels. Bertheau et al.[7] developed a sandwich technique in which suitable buffers and substrates could diffuse into acrylamide slab gels so that a variety of enzymes, including pectate lyases and polygalacturonases, could be detected in the gels.

14.32   Detecting Pectic Enzymes on Isoelectric Focusing Gels

A sensitive and rapid method for visualizing the isoelectric profiles of pectic enzymes using activity overlays has been devised.[45] Isoelectrically focused proteins in ultra-thin (0.35 mm) 5% acrylamide gels containing broad-range (pH 3.5 to 10) and high-pH (pH 9 to 11) ampholytes bonded to plastic support (Gel Bond PAG acrylamide support, FMC BioProducts, Rockland, ME 04841-2994) are overlaid with an ultra-thin (0.35 mm) layer of 1% agarose containing 0.1% polygalacturonic acid bonded on Gel Bond agarose support and also supported on plastic. After sufficient reaction time, the overlay film is stained with ruthenium red (0.05% wt/vol). The substrate gel stains a deep pink because of reaction of the ruthenium red with polygalacturonate, while the substrate in areas over the isoelectrically focused pectic enzymes has been degraded so that a clear band shows on the pink background.

14.33   Pectinesterase

Pectinesterase can be assayed based on the release of either free carboxyl groups or methanol from pectin. The most common technique is to measure the rate of release of free carboxyl groups from pectin using a pH stat.[35] A rapid, continuous spectrophotometric assay has been developed.[19] It is based on measuring the change in absorbance of a pH indicator as the pH decreases because of the formation of free carboxyl groups in pectin. Methanol may be measured colorimetrically[55] or by gas chromatography.[40] For colorimetric analysis, the modifications described by Hudson and Buescher[25] should be used because the incubation procedure of Wood and Siddiqui[55] results in nonlinear color development.

14.34   Pectate and Pectin Lyase

Pectate lyase activity can be assayed spectrophotometrically at 235 nm because of the formation of the 4,5-double bond in the nonreducing galacturonic acid residues.[51] For pectin lyase activity, pectin may be substituted for polypectate as the substrate. A pectin lyase should show little or no activity with polypectate as the substrate.

14.35   Polygalacturonase

The release of reducing groups because of hydrolysis of polypectate is the most common method for measurement of polygalacturonase activity. Lee and MacMillan[35] used 0.5% polypectate in 0.1 M sodium acetate buffer, pH 4.5 at a temperature of 30°C for tomato polygalacturonase. These are reasonable conditions for most microbial polygalacturonases, though 0.1% polypectate is usually a saturating substrate level. For measurement of reducing groups, the procedure of Nelson[42] has been used for many years. However, this method requires the addition of one reagent solution before heating and a second after heating for color development. Also, the samples must be centrifuged prior to measurement of the absorbance because of cloudiness caused by precipitated substrate. Gross[18] has demonstrated the use of 2-cyanoacetamide for the measurement of polygalacturonase activity. This reagent reacts with reducing groups to give a product that absorbs at 276 nm. Only a single addition of reagent is required, and samples do not become cloudy. Reagents are simple to prepare, and product formation is highly reproducible. The 2-cyanoacetamide reagent is preferable for most applications. However, if crude enzyme preparations have high ultraviolet (UV) absorption because of the presence of high protein concentrations, this can interfere with absorbance measurements at 276 nm. In that situation, the bicinchoninate reagent, which gives an absorption maximum at 560 nm after reaction with reducing sugars, also avoids the problems of the Nelson procedure.[39]

Decline in the viscosity of polypectate solutions has also been used to assay polygalacturonases.[10] Bell et al.[4] have described a specialized assay specifically

to assess softening activity that is due to fungal enzymes in pickle fermentation brines.

Pectic enzymes are inhibited by phenolic compounds, indoleacetic acid, fatty acid, and endopolygalacturonase end-products.[3] Tannins from certain plants inhibit both pectic enzymes and cellulase.[5] Specific protein inhibitors of polygalacturonases have also been reported in various plants. Cervone et al.[9] purified an inhibitor protein from French bean hypocotyls and demonstrated that it inhibited polygalacturonases from *Colletotrichum lindemuthianum*, *Fusarium moniliforme*, and *Aspergillus niger*.

14.4 SOURCES OF PECTIC ENZYMES

No pectic enzymes commercially available are free of other classes of pectic enzymes. The ability to clone pectate lyase genes from *E. chrysanthemi* into *E. coli* makes available clones with sequenced genes that are good producers of individual *E. chrysanthemi* pectate lyases.[31] In addition, *E. coli* clones containing *E. carotovora* genes for endo- and exo-pectate lyases are available.[46, 47] Since *E. coli* does not produce pectic enzymes, these clones will not produce pectinesterase or polygalacturonase. Phaff[44] described a yeast, *Kluyveromyces fragilis*, that reliably secretes large amounts of polygalacturonase into the growth medium. The polygalacturonase was estimated to be about 95% pure in the culture filtrate. The organism does not produce either pectinesterase or pectate lyase. Recent papers have demonstrated that three or more polygalacturonases are present in the preparation.[8, 26, 37] All of the enzymes appear to be endo-splitting with similar tissue-macerating properties.[37] Despite the presence of multiple enzymes, this is a source for easily produced polygalacturonases that are free of other classes of pectic enzymes. The culture can be obtained from Dr. Phaff for a nominal handling free.

14.5 EQUIPMENT, MATERIALS, AND REAGENTS (Chapter 62)

Crystal violet pectate (CVP) medium
Medium to detect pectate lyase (MP-7 medium)
Medium to detect polygalacturonase (MP-5 medium)
Polypectate gel medium
Polysaccharide precipitant
Selective medium for fluorescent pectinolytic pseudomonads (FPA medium)

14.6 PROCEDURES

14.61 Sample Preparation

Prepare a homogenous suspension and appropriate dilutions of the food (Chapter 2). In food containing a large amount of glucose, sufficient dilution

may be necessary to avoid possible catabolite repression of pectic enzyme synthesis.[24] Also, in high-sugar foods such as jams and jellies, exercise care in the dilution of the sample to prevent osmotic shock to the cells by the use of an appropriate buffer or isotonic solution.

14.62 Preparation and Incubation of Plates

Place not more than 0.25 mL quantities of appropriate dilutions (to obtain 20 to 30 colonies and to avoid a wet agar surface) on the surface of pre-poured plates. Distribute by a spread plate technique. Incubation of plates inoculated with a variety of plant materials has been carried out at 30°C for 48 hr. Incubation of plates inoculated with foods probably should be made at the temperature at which the food is stored. Some pectinolytic bacteria grow at 37°C, but do not produce pectate lyase until the temperature is 32°C or below. *E. carotovora* subsp. *atroseptica* often will not grow above 28°C.

14.63 Counting and Reporting

Colonies that cause depressions in polypectate gel medium or that are surrounded by a clear zone after flooding plates with a pectin precipitant are pectinolytic. Fluorescent bacteria are detected on FPA plates with long-wavelength UV light before adding pectin precipitant.

14.7 INTERPRETATION

The varieties of organisms that can degrade pectic substances are diverse. Thus, choice of the most suitable medium depends on the type of organism expected, as well as the type of food to be examined. Media and procedures are described for the detection, enumeration, and isolation of pectolytic and pectinolytic microorganisms. In addition, procedures are described for the detection of pectic enzymes. These techniques can be used to characterize degradation of fruits, vegetables, and processed foods, to screen for plant pathogens or soil organisms that degrade pectic substances, and to indicate the stability of raw plant products for storage and transport.

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COMPENDIUM

OF METHODS

FOR THE

MICROBIOLOGICAL

EXAMINATION

OF FOODS

Third Edition

Edited by:

Carl Vanderzant, PhD
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Microbiological
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