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Development of reproducible assays for polygalacturonase and pectinase

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ABSTRACT

Polygalacturonase and pectinase activities reported in the literature were measured by several different procedures. These procedures do not give comparable results, partly owing to the complexity of the substrates involved. This work was aimed at developing consistent and efficient assays for polygalacturonase and pectinase activities, using polygalacturonic acid and citrus pectin, respectively, as the substrate. Different enzyme mixtures produced by *Aspergillus niger* and *Trichoderma reesei* with different inducing carbon sources were used for the method development. A series of experiments were conducted to evaluate the incubation time, substrate concentration, and enzyme dilution. Accordingly, for both assays the recommended (optimal) hydrolysis time is 30 min and substrate concentration is 5 g/L. For polygalacturonase, the sample should be adjusted to have 0.3–0.8 U/mL polygalacturonase activity, because in this range the assay outcomes were consistent (independent of dilution factors). Such a range did not exist for the pectinase assay. The recommended procedure is to assay the sample at multiple (at least 2) dilution factors and determine, by linear interpolation, the dilution factor that would release reducing sugar equivalent to 0.4 g/L D-galacturonic acid, and then calculate the activity of the sample accordingly (dilution factor × 0.687 U/mL). Validation experiments showed consistent results using these assays. Effects of substrate preparation methods were also examined.

1. Introduction

Pectin is the plant cell wall polysaccharide with structural backbones rich in galacturonic acid residues [1,2]. Pectin contributes 2–35% of plant dry mass. It is abundant in the cell wall of growing and dividing cells, the wall of soft plant tissue, and the space between primary and secondary cell walls. The most common form of pectin is as homogalacturonan, which is a chain of α-1,4 linked galacturonic acid residues usually up to 100 residues in length. The galacturonic acid residues are commonly methylesterified or acetylated [1,3]. Other forms of pectin are broadly referred to as substituted pectin, and include rhamnogalacturonan and xylogalacturonan. Rhamnogalacturonan I, the second most common form of pectin, contains a backbone of repeating dimers of α-1,6 linked galacturonic acid and α-1,4 linked rhamnose sugars. Rhamnogalacturonans have highly varied, branched structures, usually featuring side chains of α-1,5-linked arabinan or β-1,4-linked galactan connected via the rhamnose residues. Other side chains include fucose and glucuronic acid with and without methyl substitution [1].

Pectinase is a collection of enzymes that catalyze reactions at a variety of sites on a pectin molecule. The most studied is polygalacturonase, responsible for the hydrolytic breakdown of the polygalacturonic acid backbone of pectin to galacturonic acid oligomers or monomers [4]. Another enzyme, pectin lyase, catalyzes the breakdown of pectin backbones by trans-elimination, producing an unsaturated galacturonic acid product [5]. Pectinesterase catalyzes the de-esterification of galacturonic acid residues, releasing free methanol or acetic acid [6]. Pectinesterase can increase the accessibility of pectin to polygalacturonase or pectin lyase [7]. More detailed description of the modes of action of various pectinase enzymes is available in the literature, e.g. [8].

Pectinase is used industrially to process fibers for textiles (cotton, linen), to clarify wine and fruit juices, in pulp and paper processing, and as supplements in animal feed [9–13]. While a wide range of plants produce pectinase, commercial production is usually by microbial fermentation. Pectinase is produced by fungi of the genera *Aspergillus*, *Rhizopus*, *Penicillium*, and *Neurospora*, and

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by bacteria of *Bacillus* and *Streptomyces* [14–20]. Polygalacturonase is produced by *Aspergillus*, *Saccharomyces*, *Thermoascus*, and *Fusarium* fungi and by *Bacillus* bacteria. Pectin lyase is also produced by *Aspergillus*, *Thermoascus*, *Fusarium* and *Penicillium* fungi and *Bacillus* bacteria. Pectinesterase is produced by *Aspergillus* fungi and *Erwinia* bacteria [7,21–24].

Development of accurate, consistent, and efficient assays for enzyme activities is important. Unlike assays for cellulase and xylanase activities, there were no generally accepted procedures for the assays of pectinase and polygalacturonase activities. Among various pectin-degrading enzymes, this is especially true for polygalacturonase, since the other enzymes result in chemical changes that are easier to measure: pectinesterase activity is generally determined via titration or from the pH change resulting from de-esterification of methyl ester portions of the galacturonan backbone, and pectin lyase is assayed by measuring the increase in absorbance at 235 nm that results from formation of a carbon–carbon double bond. Polygalacturonase depolymerizes the pectin backbone, and can therefore be assayed by measurement of increased sugar reducing ends or reduced viscosity [7]. Since quantitative correlation between viscosity reduction and the degree of hydrolysis was only empirical, it was decided to develop in this study an assay based on reducing end measurement, similar to the methods used for cellulase and xylanase assays.

The conditions reported in the literature for polygalacturonase and pectinase measurements vary in substrate, substrate concentration, temperature, pH, and incubation time. Substrates commonly include citrus pectin, sodium pectate, polygalacturonic acid (PGA), and galacturonic acid oligomers. The concentration varies widely between 0.09 and 1 wt%. Substrates are usually prepared in 0.05–0.1 M acetate or citrate buffers at different pH, from 4.0 to 5.5. Incubation time and temperature range from 5 to 60 min (or longer) and from 30 to 60 °C, respectively [25–30]. A summary of reported assay conditions is given in Table 1. Procedural descriptions in the literature often omit the quantity of substrate used and the enzyme activity ranges for which the assays were valid.

Because of the industrial importance of these enzymes, there was a critical need for standard methods so that the enzyme activities could be measured reproducibly and the study results compared meaningfully. In light of the inconsistent assay techniques reported in the literature, a series of experiments were conducted in this study to develop polygalacturonase and pectinase assay methods that, if possible, yield reproducible results with low sensitivities to the assay parameters. This was the first systematic study that was aimed at developing reproducible, and separate, assays for polygalacturonase and pectinase (including the use of a linear interpolation method for the pectinase assay, to eliminate the sensitivity to unknown enzyme activity in the sample analyzed, as described later in detail). These assays can be the standard methods used in the future research involving these enzymes.

2. Material and methods

2.1. Enzyme preparation

Four different enzyme mixtures were used in this study. They were produced by either *Trichoderma reesei* (NRRL 3469) or *Aspergillus niger* (NRRL 341) fermentation with different carbon sources in the media. The fermentation was made in a 3 L fermentor with 1.5 L working volume. DO (dissolved oxygen concentration) was maintained at above 20% air saturation throughout all the fermentation experiments. Fermentations #1–#3 were made with *T. reesei*; Fermentation #4 with *A. niger*. Avicel cellulose (4 g/L) and soy molasses (11.8 g/L) were the carbon source for Fermentation #1, where pH was maintained at the range of 5.3–7.0. Soy molasses are the byproduct during manufacturing soy protein products, which are rich in carbohydrates [31]. The low commercial cost disaccharide lactose (10 g/L) was the carbon source for Fermentation #2 and pH was controlled at 6 [32]. Fermentation #3 was made with 30 g/L soybean hull as carbon source and pH was 6 initially; it was allowed to drop naturally to 4 along with fungal growth and thereafter controlled at 4. Fermentation #4 was made with 40 g/L soybean hull as carbon source and pH was controlled at 5 after being allowed to drop from the initial pH 7. The nitrogen sources in the media for Fermentations #1–#3 were 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L urea and 1 g/L protease peptone; the same nitrogen sources were used for Fermentation #4 but with doubled concentrations. The other medium ingredients were kept the same for all these fermentations: 2 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.4 g/L $CaCl_2{\cdot}2H_2O$, 0.2 g/L Tween 80, and trace elements (0.005 g/L $FeSO_4{\cdot}7H_2O$, 0.0016 g/L $MnSO_4{\cdot}H_2O$, 0.0014 g/L $ZnSO_4{\cdot}7H_2O$, and 0.002 g/L $CoCl_2{\cdot}6H_2O$). The medium composition and fermentation operation were adopted and/or modified from those used previously in this laboratory for *T. reesei* fermentation [33,34]. The broths harvested at the end of fermentation (#1 192 h, #2 166 h, #3 267 h, and #4 118 h) were centrifuged (12,227 × *g* for 15 min) to collect the cell-free enzyme-containing solutions, which was stored at −20 °C for future use.

Soybean hulls and soy molasses were obtained from Archer Daniels Midland (Decatur, IL, USA). Avicel cellulose (PH-101, 50 μm particle size) was obtained from FMC BioPolymer (Philadelphia, PA, USA). All other chemical medium components were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Substrate

Polygalacturonic acid (PGA, 95% purity, average molecular weight 25,000–50,000 Da, Sigma Aldrich, product number 81325) was used as the substrate for polygalacturonase assay. Citrus pectin (galacturonic acid ≥74%, Sigma Aldrich, product number P9135) was used as the substrate for pectinase assay. Substrate solutions of 1–40 g/L were prepared in 0.1 M sodium citrate buffer at pH 4.8. The solid substrate was added gradually to the buffer while stirring to aid in formation of a uniformly opaque suspension. In the event of clump formation, the mixture was stirred for an extended period of time, sometimes with heating, to break any clumps of substrate prior to the assay. Heating was typically necessary for preparing the citrus pectin suspension. The choice of citrate buffer was based on its use in the standard assays of cellulase and xylanase. Buffer pH of 4.8 was chosen based on the reported optimal range (4.2–5.1) [28,29,35] and the optimal condition (pH 4.8 and temperature 50 °C) established in this laboratory for the intended application of these enzyme mixtures to soybean meal treatment [36].

2.3. Enzyme analysis

Polygalacturonase/pectinase activity was determined from the increase in reducing sugar concentration after incubation of the enzyme-containing sample and substrate. Reducing sugars were measured following a modified version of the dinitrosalicylic acid (DNS) method, after observing that the addition of standard DNS reagent produced a cloudy precipitate of residual substrate. This effect was more pronounced in the enzyme blanks due to their higher concentrations of intact PGA/pectin. The modified DNS reagent omitted the 30 wt% sodium–potassium tartarate in the standard recipe [37].

To perform the enzyme assay, 100 μL enzyme broth and 900 μL PGA/pectin substrate solution were added to 25 mL graduated test tubes (Corning Life Science, product number 70075-25). Blanks containing only the substrate solution were also prepared. All tubes were sealed with Parafilm and incubated in a water bath at 50 °C for times ranging from 5 to 60 min. Reaction temperature was chosen based on the reported optimum range, 40–50 °C [27,30], and the condition (50 °C) used for enzymatic hydrolysis of carbohydrate in soybean meal. The enzyme reaction was terminated by addition of 3 mL of a modified DNS solution that consisted of 10 g/L 3,5-dinitrosalicylic acid and 16 g/L sodium hydroxide (NaOH). Enzyme broth was then added to the blanks, to account for the turbidity introduced with the enzyme broth. All samples were boiled for 10 min to develop color, diluted to a total volume of 25 mL, and inverted several times to mix. The absorbance values were measured at 540 nm and calibrated against standard solutions of D-galacturonic acid·H_2O at 0, 0.125, 0.25, 0.5, and 1 g/L. The final activity calculation was based on the release rate of galacturonic acid-equivalent reducing sugar from the PGA/pectin substrate. One unit (U) is equivalent to one μmol product released per min. The enzyme activity (U/mL) is calculated according to the following equation:

$$\text{Activity(U/mL)} = \frac{\mu\text{g galacturonic acid released}}{0.1 \times 194.1 \times t} \quad (1)$$

where "0.1" mL is the enzyme broth volume used in the assay, "194.1" is the molecular weight of galacturonic acid, and *t* is the reaction time in min.

Based on these test procedures and conditions, a series of experiments were designed to evaluate the effects of reaction time, substrate concentration, and enzyme dilution on polygalacturonase assay. First, undiluted enzyme solution from Fermentation #1 was incubated with 10 g/L PGA solution for 5–60 min to determine a suitable reaction time. Next, the effect of substrate concentration ranging from 1 to 40 g/L was studied in 3 sets of experiments using the enzyme solutions from Fermentations #1 and #2, with different dilution factors, to determine the substrate concentration to use for the assay. Then, multiple dilutions of the enzyme solution from Fermentation #3 were assayed with the determined PGA concentration to evaluate the range of enzyme activities that would give consistent results. The enzyme solution from Fermentation #3 was also used to validate the established reaction time and substrate concentration for polygalacturonase assay. Finally, the same assay conditions were evaluated and modified for applicability to pectinase