Validation of leaf and microbial pectinases: commercial launching of a new platform technology

Henry Daniell1,* Thuanne Ribeiro1, Shina Lin1, Prasenjit Saha1, Colleen McMichael2, Rashmi Chowdhary1 and Anshika Agarwal1

1Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA
2Phyllozyme, Pennovation Center, Philadelphia, PA, USA

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*Correspondence (Tel 215-746-2563; fax: 215-898-3695; email: hdaniell@upenn.edu)

Summary
Almost all current genetically modified plant commercial products are derived from seeds. The first protein product made in leaves for commercial use is reported here. Leaf pectinases are validated here with eight liquid commercial microbial enzyme products for textile or juice industry applications. Leaf pectinases are functional in broad pH/temperature ranges as crude leaf extracts, while most commercial enzyme products showed significant loss at alkaline pH or higher temperature, essential for various textile applications. In contrast to commercial liquid enzymes requiring cold storage/transportation, leaf pectinase powder was stored up to 16 months at ambient temperature without loss of enzyme activity. Commercial pectinase products showed much higher enzyme protein PAGE than crude leaf extracts with comparable enzyme activity without protease inhibitors. Natural cotton fibre does not absorb water due to hydrophobic nature of waxes and pectins. After bioscouring with pectinase, measurement of contact-angle water droplet absorption by the FAMAS videos showed 33 or 63 (leaf pectinase), 61 or 64 (commercial pectinase) milliseconds, well below the 10-second industry requirements. First marker-free lettuce plants expressing pectinases were also created by removal of the antibiotic resistance aadA gene. Leaf pectinase powder efficiently clarified orange juice pulp similar to several microbial enzyme products. Commercial pilot scale biomass production of tobacco leaves expressing different pectinases showed that hydroponic growth at Fraunhofer yielded 10 times lower leaf biomass per plant than soil-grown plants in the greenhouse. Pectinase enzyme yield from the greenhouse plants was double that of Fraunhofer. Thus, this leaf-production platform offers a novel, low-cost approach for enzyme production by elimination of fermentation, purification, concentration, formulation and cold chain.

Keywords: chloroplast enzymes, ambient storage, bioscouring, fruit juice, pectinase, stability, textile.

Introduction
Pectins are categorized as soluble or insoluble fibres, which cannot be absorbed by the human digestive system. However, pectinases digest pectin by modifying them to short polysaccharide fragments that can be absorbed thereby enhancing human nutrition. Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galacturonic acid residues. Pectinases break down the glycosidic linkages at C-4 and simultaneously eliminate H from C-5, producing a D 4:5 unsaturated product from pectins. They are used in the fruit-processing industry to improve production efficiency by enhancing clarification/liquefaction to reduce viscosity and increase filterability of juices, and enzymatic maceration/extraction of plant cells to release flavour, nutrients, vitamins, proteins and carbohydrates (van den Broek et al., 1997; Joshi et al., 2011; Lee et al., 2006; Sharma et al., 2017).

Pectin is a major non-cellulosic impurity (~10%) of cotton fibres located mainly in the cuticle, primary cell wall and middle lamella. The hydrophobic nature of waxes and pectins decreases water absorption of native cotton fibres that impedes uniform and efficient dyeing and finishing that are done under aqueous conditions (Lin and Hsieh, 2001). In order to address these challenges, pretreatment processes use harsh chemicals, alkaline pH and other severe conditions that are problematic for our environment, through release of toxic effluents from textile plants resulting in their relocation to countries with less stringent regulations. Water absorption is among the most important quality of cotton fabrics, and yet, natural cotton fibre is highly inefficient in absorbing water. In order to address these concerns, pectinases are used for textile bioscouring to enhance water absorbency of cotton fibres, without causing cellulose destruction. Further enhancement of wettability through bioscouring can be achieved using the combination of pectinase with lipase, xylanase and other cellulases (Kalantzi et al., 2008). Bending and shear rigidity, extensibility and compressional resilience are also improved by pectinase treatment (Kalantzi et al., 2008).

Aforementioned applications require large quantities of enzymes. However, costs of current microbial enzymes are prohibitive and limit their extensive use in various industrial biological applications. Decades-old microbial production systems require prohibitively expensive fermentation facilities, purification from host cells, formulation to increase concentration and stability and cold storage/transportation. Therefore, there is a great need to explore novel production technologies that could eliminate these prohibitively expensive enzyme processes. While the cost of enzyme production, mark-up by industries and production capacity are often debated, launching of leaf enzyme products, through PhylloZyme funded by investors who own
textile or microbial enzyme industries, underscores the need for new platform technologies.

Although we have previously reported expression of pectinases in leaves (Verma et al., 2010), and other groups reported microbial diseases associated with release of pectinase (Ballan et al., 2018; Shirsath et al., 2018), no leaf-made enzyme product has been launched commercially nor validated with commercial microbial enzyme products. Almost all genetically modified (GM) products are derived from seeds. Therefore, in this study, we validate pectinases produced in tobacco or lettuce leaves with eight commercial liquid microbial enzyme products (Table 1) for the textile or food industries that were available for testing. Pectinases were evaluated using commercial validation processes including contact-angle water droplet absorption through the FAMAS bioscouring videos or fruit juice clarification. Commercial-scale production of leaf biomass and pectinase yield were compared in hydroponic- and soil-growth facilities. Crude extracts of leaf enzyme powder (stored for 16 months at ambient temperature) were compared with commercial microbial pectinase products for temperature and pH optima, without need for stabilizing agents or formulation. This is the first report of commercial leaf enzyme products.

Table 1: Commercial pectinase products used in this study, their manufacturer/supplier, origin of transgene expressed, and storage format and temperature requirements

| Sample                  | Company                | Organism                   | Solid/Liquid | Storage |
|-------------------------|------------------------|----------------------------|--------------|---------|
| Chloroplast-derived     | PhyloZyme              | Fusarium solani            | Solid        | Ambient |
| PeIA                    |                        |                            |              |         |
| Chloroplast-derived     | PhyloZyme              | Fusarium solani            | Solid        | Ambient |
| PeIB                    |                        |                            |              |         |
| Chloroplast-derived     | PhyloZyme              | Fusarium solani            | Solid        | Ambient |
| PeID                    |                        |                            |              |         |
| Pectin Lyase (PL LIQUID)| Specialty Enzymes &   | Bacillus                   | Liquid       | 4 °C    |
|                         | Biotechnologies        | licheniformis ?            |              |         |
| Polyalgalacturonase     | Specialty Enzymes &   | Bacillus                   | Liquid       | 4 °C    |
| (PG LIQUID)             | Biotechnologies        | pumilus ?                  |              |         |
| Pectin Methyl Esterase  | Specialty Enzymes &   | Aspergillus niger          | Liquid       | 4 °C    |
| (PME LIQUID)            | Biotechnologies        |                            |              |         |
| Pectate Lyase R80L      | Specialty Enzymes &   | Bacillus sp ?              | Liquid       | 4 °C    |
| (ClariSEB R80L)         | Biotechnologies        |                            |              |         |
| Pectinase               | Biogreen               | Bacillus subtilis ?        | Liquid       | 4 °C    |
| (Bioprime Scour Ultra)  |                        |                            |              |         |
| Pectinase 260L          | Enzyme Supplies        | Aspergillus niger          | Liquid       | 4 °C    |
| Bioprep 3000L           | Novozymes              | Bacillus subtilis ?        | Liquid       | 4 °C    |
| Alkaline Pectinase      | Sinobios               | Bacillus subtilis ?        | Liquid       | 4 °C    |

Organisms with ? are predicted from protein size.

Results

Temperature and pH optima of crude leaf extracts and commercial enzyme products

Pectinase expressed in tobacco chloroplasts (Cp) was evaluated in crude leaf extracts from plant powder stored for more than one year at ambient temperature. CpPelA, CpPelB and CpPelD were harvested, lyophilized and stored at ambient temperature for 14, 15 or 16 months before investigations. Commercial pectinases were not chosen based on any specific criteria, and all pectinases that could be obtained from different sources were tested (Table 1). All pectinases obtained were in liquid form and stored at 4 °C. Some batches of liquid commercial enzymes showed microbial contamination after long-term storage and were replaced with new batches. Testing was based on enzyme equivalency and not based on weight or protein concentration because commercial product packages did not report enzyme name, units, concentration or details of formulation (non-enzyme products or stabilizing agents or filler materials).

All tested leaf and microbial pectinases showed highest activity at pH 8.0 (Figure 1a,b). CpPeIA, CpPeIB and CpPeID enzyme activity observed was exclusively due to engineered PeIA, B and D genes because no measurable pectinase activity was observed in untransformed WT leaf extracts. Pectinase is used for bioscouring applications at alkaline pH. At pH 10, CpPeIA and CpPeID retained 87%–88% of activity, while most commercial pectinases lost significant enzyme activity. For example, at pH 10, Alkaline Pectinase® (Sinobios, Shanghai, China), Pectinase 260L® (Enzyme Supplies, Oxford, UK) and Bioprep® 3000L (Novozymes, Franklinton, NC) retained 30%, 33% and 46% pectinase activity, respectively. Higher activity of CpPeIA and CpPeID (with loss of 12–13% activity) could be due to the origin of pelA and pelD genes from Fusarium solani. At pH 10, Pectin Lyase from Specialty Enzymes & Biotechnologies, derived from Aspergillus niger, retained 72% of activity. However, Pectinase from Biogreen®, derived from Bacillus subtilis, showed 91% of activity at pH 10. Similar to chloroplast pectinases (87–88%) and this is the best alkaline enzyme among commercial pectinases tested in this study. It is ironic that enzymes with the ‘alkaline pectinase’ designation retains <30% activity at pH 10. Therefore, both chloroplast and commercial enzymes were analysed in three independent biological samples across all data points and observed results are significant and reliable.

All tested plant and microbial pectinases showed highest activity at 40 °C (Figure 2a,b). Only CpPeIA, CpPeIB and CpPeID and Pectinase 260L® showed 98–100% pectinase activity at 50 °C, while all other commercial enzyme products showed significant decline in activity at higher temperatures. CpPeIA maintained 68%, CpPeIB 69% and CpPeID 74% activity at 80 °C, while Alkaline Pectinase showed 40%, Pectin Lyase 51%, Polyalgalacturonase 45%, ClariSEB R80L 48% and Pectinase 260L® 46%. Pectinase 260L maintained 73% activity similar to CpPeIA.

Comparison of stability of crude leaf extracts with commercial enzyme products

Through the Coomassie-stained gel, it can be seen that there is not much degradation in plant proteins without any protease inhibitors (Figure 3a) from lyophilized plant cells when stored for more than one year at room temperature. This is in sharp contrast to the requirement of cold storage for all tested microbial liquid
enzymes which require hyper-concentration and elimination of proteases through purification or formulation with protease inhibitors. A unique polypeptide is observed at ~28 kDa in CpPelA, CpPelB and CpPelD crude plant extracts but absent in untransformed leaf extracts. Differences in protein size for the different microbial enzymes (Figure 3b) are due to their diverse origin. Most microbial industry products showed high quantity of pectinases except Pectin Methyl Esterase and Pectinase 260L, although >50% pectinase activity was observed. A smaller ~28 kDa polypeptide was observed in Polygalacturonase and ClariSEB R80L which are probably derived from Aspergillus niger. Pectinase (Biogreen Enzyme, Sumutprakarn, Thailand) and Pectin Lyase from Specialty Enzymes & Biotechnologies, Chino, CA showed ~30 kDa proteins. Bioprep 3000L, Alkaline pectinase showed the largest protein at ~38 kDa because they are probably derived from Bacillus subtilis. None of the commercial packages provided details on the origin of enzyme (genus, species or strain), and therefore, aforementioned sources are based on protein sizes for pectinases reported in published literature. Pectinase concentration (band intensity) was quantified in Coomassie-stained gels, and quantified polypeptides are identified in red boxes; there was 3.94–4.56 pectinase activity in crude CpPel A, B and D extracts while there was 27.20–28.58 enzyme units in commercial microbial enzymes. However, polypeptide band intensity varied 6.9–7.9 in crude plant extracts but 320–456 in commercial products, with 46–58-fold higher density (Figure 3a,b). Because antibody was not available, these differences could not be quantified more accurately, but in general, much higher microbial enzyme load was observed for similar level of activity. Elimination of purification steps and subsequent formulation to enhance

Figure 1  (a) Comparison of pH optima of leaf crude extracts with commercial pectinase products. Lyophilized powder (20 mg) of CpPelA, CpPelB and CpPelD was extracted in 200 μL Tris-HCl buffer (50 mM, pH 8.0), and 15 μL was used in each assay. Commercial liquid enzymes (1 μL): Pectinase 260L (Enzyme Supplies), Pectinase (Biogreen), Bioprep 3000L (Novozymes) and Alkaline Pectinase (Sinobios). The substrate (50 μL of 0.25% Polygalacturonic acid) was dissolved in Tris-HCl buffer (50 mM), and 10 min incubation was performed at temperature (40°C) at different pH (4–11). WT untransformed plant is used as the negative control. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation. (b) Comparison of pH optima of leaf crude extracts with commercial pectinase products. Lyophilized powder (20 mg) of CpPelA, CpPelB and CpPelD was extracted in 200 μL Tris-HCl buffer (50 mM, pH 8.0), and 15 μL was used in each assay. Commercial liquid enzymes (1 μL): ClariSEB R80L, Pectin Methyl Esterase, Polygalacturonase, Pectin Lyase (Specialty Enzymes & Biotechnologies). The substrate (50 μL of 0.25% Polygalacturonic acid) was dissolved in Tris-HCl buffer (50 mM), and 10 min incubation was performed at temperature (40°C) at different pH (4–11). WT untransformed plant is used as the negative control. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.
stability and cold storage/transportation offers unique advantages of plant crude extracts stored at ambient temperature for several months.

Bioscouring of cotton fabric using crude leaf extracts or commercial enzyme products

Bioscouring of cotton fabric was performed under conditions currently used by the textile industry. Bioprep 3000L (Novozymes) was compared with CpPelA, using water and untransformed WT as negative controls. After 60 min of bioscouring in 25 mL water in 100 mL beakers at 60 °C, textile samples were washed at 80 °C for 10 min, dried at room temperature, and water absorption of textile was evaluated by the ‘drop test’. Visually (Figure 4a, top panel), it is evident that there was no water absorption in the white textile in negative controls at pH 8.5, but Bioprep 3000L and CpPelA (crude extract) showed rapid absorption (note water droplet or absorbed water within marked circles). For measurement of contact angle and droplet absorption, FAMAS analysis software, Kyowa Interface Science, Niiza, Japan was used (Figure 4a bottom panel, Videos S1–S4). A significant difference in absorption between tested samples was observed, but CpPelA showed rapid absorption like the microbial enzyme. Through the analysis of the FAMAS software, it was observed that CpPelA increased the area of contact between the cotton surface and the aqueous layer, allowing the absorption of water. The FAMAS videos showed full absorption at 33 ms for

Figure 2  (a) Comparison of temperature optima of leaf crude extracts with commercial pectinase products. Lyophilized powder (20 mg) of CpPeIA, CpPeIB and CpPeID was extracted in 200 µL Tris-HCl buffer (50 mM, pH 8.0), and 15 µL was used in each assay. Commercial liquid enzymes (1 µL): Pectinase 260L, Pectinase, Bioprep 3000L and Alkaline Pectinase. The substrate (50 µL of 0.25% Polygalacturonic acid) was dissolved in Tris-HCl buffer (50 mM), and 10 min incubation was performed at pH (8.0) at different temperatures (30–90 °C). WT untransformed plant is used as the negative control. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation. (b) Comparison of temperature optima of leaf crude extracts with commercial pectinase products. Lyophilized powder (20 mg) of CpPeIA, CpPeIB and CpPeID was extracted in 200 µL Tris-HCl buffer (50 mM, pH 8.0), and 15 µL was used in each assay. Commercial liquid enzymes (1 µL): ClariSEB R80L, Pectin Methyl Esterase, Polygalacturonase, Pectin Lyase. The substrate (50 µL of 0.25% Polygalacturonic acid) was dissolved in Tris-HCl buffer (50 mM), and 10 min incubation was performed at pH (8.0) at different temperatures (30–90 °C). WT untransformed plant is used as the negative control. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.

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CpPelA at pH 8.5 when compared to 61 ms for Bioprep 3000L, both exceeding the current industry standard of 10 s (Videos S3–S4, to be played in Windows Media Player). At pH 10, absorption of water was almost similar for CpPelA (63 ms) and Bioprep 3000L (64 ms, videos not shown).

In order to correlate bioscouring efficiency with pectinase activity, enzyme activity was monitored in three enzyme aliquots before and after (60 min) for each beaker experiment (Figure 4b). CpPelA activity was slightly higher than Bioprep 3000L, both before or after the bioscouring test. While enzyme stability is anticipated in microbial preparations because they are free of proteases and formulated with stabilizing agents, stability of pectinase CpPelA in crude extracts in alkaline pH at 60 °C for more than 1 h in water was surprising. Loss of pectin from the surface of the fabric during bioscouring was slightly greater in CpPelA than Bioprep 3000L (Table 2), which may correlate with the slightly higher pectinase activity in CpPelA.

**Marker-free lettuce chloroplasts expressing Pel B and Pel D enzymes**

The marker-free chloroplast vector was generated by multiple cloning steps comprising the spectinomycin-resistant aminglycoside-3'-adenyllyl-transferase (aadA) gene under the control of the plastid ribosomal RNA promoter (Prom) and followed by the 3'UTR, TrbcL. The aadA gene is located between two copies of plastid-encoded CF1 ATP synthase subunit beta (atpB) promoter region (649 bp, Figure 5a, Upper left panel). The coding sequences, including pelB or pelD, were inserted into pLsLF-MF, under the control of the p5bA promoter/5' UTR and 3' UTR (Figure 5a). After bombardment (4–6 weeks), primary regenerated shoots directly grew from the leaf explants without formation of callus on spectinomycin-containing media (Figure 5b).

During the selection process on spectinomycin-containing RMOP medium, the aadA cassette is excised, releasing one copy of the atpB region in the transplastomic genome by homologous recombination between the two directly repeated atpB fragments. Once the homology-based marker excision happens, 16S-Forward/atpB-Reverse primers amplified the 2.4-kb PCR product (Figure 5c–Lanes 1–4 (PelB) and Lanes 1–5 (PelD) middle panel) and the 16S-Forward/aadA-Reverse primers did not produce any PCR product (Figure 5c, top panel). When the transplastomic genome contained the intact expression cassette, a 4.4-kb DNA fragment should be produced in addition to the 2.4-kb product. Site-specific integration at the 3' end of the transgene cassette is confirmed by a 2.4-kb PCR product amplified by the UTR-F/F23s-R primer (Figure 5c, PelB—Lanes 1–4 and PelD—Lanes 1–5). Lettuce plants showed site-specific integration of transgene cassettes containing pelB or pelD genes in all tested lines and removal of the aadA gene only in a few transplastomic lines.

Transplastomic lines grew normally (Figure 5b) and set seeds. Lyophilized lettuce CpPelB showed eightfold and CpPelD sevenfold higher pectinase activity than fresh lettuce leaves based on leaf weight (Figure 5d,e). Remarkably, lettuce pectinases showed activity in a broad pH and temperature range (Figure 5d,e), making them suitable for a variety of applications. Bioscouring activity is performed at pH 8.5 or 10.0 while juice clarification is performed at pH 5.5. Lettuce pectinase shows 94% activity at pH 8.5 or 10.0 and 95% at pH 5.0 (Figure 5e). Likewise, juice clarification is done at 50 °C and bioscouring at 60 °C; lettuce pectinase maintains 85% activity at these temperatures (Figure 5d). Therefore, transplastomic lettuce pectinases are suitable for several food/feed or industrial applications.

**Clariﬁcation of orange fruit juice by crude leaf extracts or puriﬁed commercial enzyme products**

The efficacy of the enzymes in the clarification process was evaluated by measuring transmittance (A700) in triplicate across each time point during the assay. Experimental samples were incubated at 50 °C, pH 5.5 with shaking at 150 rpm. Aliquots (100 μL) taken at different time points (0, 30, 60, 90, 120 min) were measured at A700. Gradual clarification was observed based
on duration of incubation (Figure 6a), reaching almost zero after 2 h. The initial rates of chloroplast pectinase clearance activities were faster than at later time points, but gradual clarification continued until 120 min. No clarification was observed in the blank (water) and untransformed WT leaf extracts at A 700 (Figure 6b) or by visual examination. Chloroplast pectinases showed better clarification than some microbial enzymes like Polygalacturonase (PG) and Pectin Lyase (PL) and was similar to Bioprep 3000L and Pectinase 260L. Again, correlation between pectinase activity and fruit juice clarification was investigated by measuring galacturonic acid release (Figure 6c). Chloroplast pectinases showed slightly higher activity than the microbial enzymes Polygalacturonase (PG) and Pectin Lyase (PL) and similar when compared with Bioprep 3000L or Pectinase 260L. Again, stability at 50 °C of chloroplast pectinases is remarkable because crude extracts contain abundant proteases and no protease inhibitors were used to stabilize pectinases in leaf extracts.

Large-scale biomass production

All materials used in investigations described above were from plants grown in the Daniell laboratory greenhouse and stored for 14 (CpPelA), 15 (CpPelB) and 16 (CpPelD) months after lyophilization. Figure 7a,b shows data comparing biomass yields from the greenhouse or hydroponic Fraunhofer production systems. CpPelA, B, and D biomass yield per plant from Fraunhofer based on harvest varied from batch to batch from

Figure 4 (a) Evaluation of bioscouring of cotton fabric using crude leaf extracts or Bioprep3000L (Novozymes): In 25 mL water, 100 µL of enzyme was added and incubated for 60 min at 60 °C. After draining water, fabric was washed at 80 °C for 10 min and air dried. WT untransformed plant or water blank was used as the negative controls. Top panel: pH 8.5/60°C and pH10/60°C water. Bottom panel: Drop test was evaluated at pH 8.5 using FAMAS analysis software. See Videos S1–S2 (negative controls), Video S3 (Bioprep) and Video S4 (Cp-PelA). (b) Enzyme assay before and after bioscouring: Substrate (Polygalacturonic acid 0.25%) was dissolved in Tris-HCl buffer (50 mM, pH 4.0–11.0) and incubated at 40 °C for 10 min. WT untransformed plant or water blank was used as the negative controls. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.
3.12 to 13.06 g fresh weight (FW). In the greenhouse, CpPelB FW per plant increased from 12 g FW in 5 weeks to 96 g in 16 weeks; CpPelD increased from 12 g FW in 6 weeks to 107 g FW in 14 weeks. Enzyme activity was higher for pectinases (85 708 \( \text{l mole per hour per gram fresh weight for CpPelA,} \) 78 444 for CpPelB and 54 607 for CpPelD) grown in the greenhouse (Figure 7d) than Fraunhofer (40 127 for CpPelA, 35 294 for CpPelB and 34 238 for CpPelD; Figure 7c). Figure 7a, b shows that all plants grown in the greenhouse are much larger than those grown at Fraunhofer. Enzyme yield of pectinase was also higher for greenhouse than Fraunhofer plants on a fresh-weight basis (Figure 7c,d).

### Discussion

Leaves are grown throughout the plant growth cycle in contrast to seeds produced at the tail end of the growth cycle. However, mostly seeds are used as commercial products and no high-value products are produced in leaves, even though they could contain very high levels of proteins (e.g. Amaranthus 57.8%, cowpea 29.8% DW—Nagy et al., 1978; Nell and Siebrits, 1992). The most abundant protein on earth is the leaf protein Rubisco, made in chloroplasts (Cosa et al., 2001). Therefore, we explore here the feasibility of producing high-value enzyme products in high-biomass tobacco leaves for non-food/feed industrial applications and lettuce leaves for edible food/feed applications. The few enzyme products engineered via the nuclear genome and expressed in corn seeds (Park et al., 2016) require purification and storage/transportation, similar to microbial enzymes (e.g.

Table 2: Weight loss of cotton fabric as a result of bioscouring

| Sample | Weight before (g) | Weight after (g) | Final weight lost (g) |
|--------|------------------|-----------------|----------------------|
| WT (pH 8.5) | 0.1186 | 0.1170 | 0.0016 |
| WT (pH 10.0) | 0.1222 | 0.12 | 0.0022 |
| Water (pH 8.5) | 0.1278 | 0.12 | 0.0078 |
| Water (pH 10.0) | 0.1186 | 0.116 | 0.0026 |
| Bioprep 3000L (pH 8.5) | 0.1242 | 0.114 | 0.0102 |
| Bioprep 3000L (pH 10.0) | 0.1252 | 0.1103 | 0.0149 |
| CpPelA (pH 8.5) | 0.1373 | 0.1201 | 0.0172 |
| CpPelA (pH 10.0) | 0.1268 | 0.113 | 0.0138 |

Figure 5 (a–e) Marker-free lettuce transplastomic lines expressing pectinases. (a) Schematic representation of the lettuce chloroplast 16S trnI/trnA region, chloroplast expression cassette containing pectinase pelB or pelD transgene cassette (b) Primary regeneration in lettuce without formation of callus, rooting in Magenta box and grown in the greenhouse. (c) PCR product of size 2.439 kb with primer set 16S−F/atpB−R and 2.454 kb with TpsbA−F/23S−R confirms integration of cassette into lettuce chloroplast genome; absence of PCR product with 16S−F/3M−R primer sets confirms removal of marker gene from the transplastomic plants. (d) Evaluation of pectinase in fresh (200 mg/600 \( \text{L} \)) and lyophilized (20 mg/200 \( \text{L} \)) lettuce cells at different temperature (30–90 °C) and (e) pH (4.0–11.0) using polygalacturonic acid substrate. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.
Figure 6 Clarification of orange fruit juice by crude leaf extracts or commercial pectinase products: (a) 100 µL of Bioprep 3000L, Pectinase 260L, Polygalacturonase (PG) and Pectin Lyase (PL) were compared with CpPelA, CpPelB and CpPelD at 50°C, pH 5.5 and 150 rpm. Samples were centrifuged at 5000 × g for 15 min. (b) Supernatant before or after centrifugation was measured at A700 at indicated times. (c) Pectinase activity in fruit juice before and after clarification. The substrate (Polygalacturonic acid 0.25%) was dissolved in Tris-HCl buffer (50 mM, pH 4.0–11.0), and incubation (10 min) was performed at 40 °C. WT (untransformed plant) and water blank served as negative controls. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.
Infinite Enzymes). Although there is one publication on expression of pectinases in leaves (Verma et al., 2010), we have not located any leaf enzyme commercial product. To the best of our knowledge, this is the first report of engineered high-value commercial products produced in leaves for industrial applications using crude leaf extracts (Table 1; Figures S1 and S2).

Chloroplast genetic engineering offers several unique advantages including high-level expression (Bally et al., 2009; Clarke et al., 2017; Daniell et al., 2016a,b; Jin and Daniell, 2015; Sanz-Barrio et al., 2011), transgene containment through maternal inheritance (Daniell, 2002, 2007) and production in leaves enabling harvest before flowering offer almost complete transgene containment. Biomass for enzyme products is currently produced in Fraunhofer hydroponic facility by PhylloZyme. As discussed below, biomass yield per plant is 10-fold lower from Fraunhofer hydroponic system than in the greenhouse. Much higher biomass production will be needed in metric ton quantities of enzymes required for food, feed, juice, textile, brewery, detergent, paper, pulp, waste water treatment, bioethanol and various other industries. Cost of enzymes for cellulosic ethanol industry (Latarullo et al., 2016). For such large-scale biomass production, field production may be necessary. In this regard, products made in chloroplasts have been grown in the field almost a decade ago with USDA-APHIS approval (Arlen et al., 2007). However, a recent USDA-APHIS notice (Kwon and Daniell, 2015) states that transplastomic lines do not fit the definition of a regulated article under USDA-APHIS regulations 7 CFR part 340, because there are no plant pest components, which should further help in advancing this technology.

In this study, leaves were dried through a lyophilization process because this process has been optimized for biopharmaceuticals expressed in leaves (Daniell et al., 2016a,b; Su et al., 2015). However, for commercial-scale production, other low-cost approaches should be investigated. Protein drugs produced in leaves are highly stable when plants were dried in the greenhouse without watering (Boyhan and Daniell, 2011). Xylanase produced in leaves retained full activity when they were dried in the sun (Leelavathi et al., 2003). In the section below, technologies currently used for drying tobacco leaves are discussed. Chen et al. (2019) recently reported processing of tea leaves to preserve quality and aroma; freshly plucked tea leaves were naturally dried on bamboo sieves at 21–24 °C, with 65–82% humidity, for 48 h. Dried tea leaves were further dehydrated at 60 °C for 2 h to obtain the final tea product. However, none of the commercial drying processes evaluate protein quality or quantity because

![Figure 7](image_url)
there is no commercial protein product made in leaves. Therefore, drying process needs to be optimized to maintain enzyme activity. Because pectinase is stable at 60 °C in water, adopting tea drying process to lettuce or tobacco by PhyloZyme should not pose a major challenge.

Enzymes produced in leaf dry biomass in this study are stable at room temperature for several months and require no purification or unique formulation for stability. In lyophilized leaves, enzymes were stable even after storage for 16 months. However, enzyme stability at higher temperature or acidic or alkaline pH during bioscouring is a feature of that specific protein and not due to their expression in chloroplasts. The presence of hundreds of plant proteases in crude extracts did not degrade chloroplastic pectinases, and their activity was maintained after long incubations at alkaline pH or higher temperatures, without exogenous addition of protease inhibitors. Commercial concentrated enzymes used in this study are heavily protected by formulations that include protease inhibitors, and microbial proteases have been eliminated through expensive purification processes. Most strikingly, microbial commercial pectinases are more highly concentrated than in crude leaf extracts, when equal enzyme units were resolved in SDS-PAGE gels. This implies that purified microbial products are needed in much higher concentrations than those produced in plants to perform the same function. In addition, long-term storage in lyophilized plant cells at ambient temperature is conferred by bioencapsulation of enzymes within plant cell wall, which is not feasible with microbial commercial pectinase products. Indeed, long-term storage of commercial microbial liquid enzyme products resulted in microbial contamination when stored at 4 °C.

Leaf biomass yields from our study follow a general trend that more leaf material can be harvested from older plants, with some fluctuations that may be due to the subjective nature of our leaf selection for harvest. Leaf biomass yield from the greenhouse exceeded that from Fraunhofer around 10-fold, likely due to the crowding of *N. tabacum* in a hydroponic system designed for much smaller plants. Pectinase activity was detected at more or less steady levels in leaves collected throughout the life span of hydroponically grown plants, with average activity levels slightly higher in CppelA than CppelB or D. In contrast, greenhouse-grown plants showed higher activity overall in all lines as compared to Fraunhofer, with CppelA exceeding both CppelB and D. Greenhouse CppelA, like that of Fraunhofer, showed steady activity throughout the life of the plant. Taken together, these data show that the greenhouse has the most optimal conditions for Cppel leaf biomass generation in general, and that CppelA in particular shows optimal yield of enzyme activity.

Bioscouring of cotton fabric yielded the most interesting results through contact angle and droplet absorption in FAMAS analysis. The wettability of the fabric can be measured by calculating the contact angle between the surface and the water droplet. When a droplet is deposited onto a solid substrate, the liquid forms a drop shape. The point where the solid, the liquid and vapour meet is called the three-phase point, and it determines the contact angle. The lower the angle between the surface and the drop, the better the wettability (Agrawal et al., 2008; Kurusu and Demarquette, 2018; Nowak et al., 2013). The absorption of water on the substrate was analysed using a contact angle analyser by Kyowa (DMe211 model, Figure S3). Visual observation showed that there was no water absorption in natural cotton fibre (negative controls) due to waxes or pectins but bioscouring with Bioprep 3000L or CppelA showed rapid water absorption. The FAMAS videos showed 32 or 63 ms for CppelA when compared to 61 or 64 ms for Bioprep 3000L at pH 8.5 or pH 10.0, well below the current industry standard of 10 s. Bioscouring efficiency correlated very well with pectinase activity, which was stable after 1 h at alkaline pH and high temperature. Loss of pectin from the surface of the fabric during bioscouring was slightly higher in CppelA than Bioprep 3000L, which also correlated with higher pectinase activity.

Pectinases are also used in the fruit-processing industry to enhance clarification/liquefaction and increase filterability of juices, releasing flavour, nutrients, vitamins, proteins and carbohydrates. Increase in clarification using fungal pectinases was reported for lychee, apricot, banana, apple, grape and passion fruit juices (Joshi et al., 1991; Mantovani et al., 2005; Sharma et al., 2017). Citrus fruit juices contain colloids that are mainly polysaccharides (pectin, cellulose, hemicellulose, lignin and starch), protein, tannins and metals (Ucan et al., 2014). Enzymatic juice clarification processes reduce viscosity and enhance juice extraction, which is further separated using centrifugation and filtration. Cocktails for citrus fruit juice clarification mainly contain pectinases, cellulases, hemicellulases and amylase (Sharma et al., 2017). In this study, we observed that leaf pectinases are equally or more efficient in clarification than commercial juice processing products but have much lower cost of goods because of elimination of cold chain, fermentation and purification processes. However, other enzymes are needed to break down lignin, cellulose, hemicellulose and xylan to achieve total clarification and future studies will include the synergistic effect of enzymes.

The plant chloroplast system has been advanced to express therapeutic proteins with various advantages including high-level expression and ease of oral administration through edible crops (Herzog et al., 2017; Kwon et al., 2016, 2018). Although FDA-approved GM edible crops have been in use for more than two decades, all of them contain antibiotic resistance genes. Currently, there is no GM crop free of antibiotic resistance genes in the marketplace. There is also no GM crop producing enzymes free of antibiotic resistance genes. However, the retention of the antibiotic resistance gene in transplastomic lines could pose hurdles in the regulatory approval process, probably because of larger copy numbers in each cell. Furthermore, elimination of the antibiotic resistance genes not only reduces metabolic load of the transplastomic crops but also enables the same selection marker to be reused for subsequent transformation of additional genes. Therefore, in the expression cassette containing the pelB and pelD genes, we used 649 bp of two atpB promoter regions to facilitate marker gene excision from the lettuce chloroplast genome, following the method developed by Day’s group for marker removal from the tobacco chloroplast genome (Day and Goldschmidt-Clermont, 2011; Lamtham and Day, 2000; Kode et al., 2006). Lettuce plants showed site-specific integration of transgene cassettes containing pelB and pelD genes. Transplastomic lines grew normally and set seeds. PelB and PelD enzyme produced in lettuce leaves show enzyme characteristics (optimal pH, temperature) suitable for various applications in the juice and textile industries. Availability of these enzymes in an edible crop offers the opportunity for advancing the food/feed applications of enzymes.

There are several reasons for projected low-cost production for enzymes produced in leaves. Production costs of dried (cured) tobacco leaves have varied between $1.48 and 1.85/lb in
the past decade, and operating and machinery costs are $3,213 per acre. (https://tobacco.ces.ncsu.edu/wp-content/uploads/2012/07/tobacco-production-cost-2011-1.pdf?w=960). Therefore, tobacco leaf biomass production cost is much lower than that of any microbial fermentation facility construction, operation and maintenance. In addition, high costs of microbial enzyme purification or enrichment, formulation, stabilization, cold storage and transportation are completely eliminated because enzymes in dried leaves are fully functional, without need for these expensive purification or formulation processes. Most early applications of pectinase are aimed at minimal processing for direct bulk use of leaf powder in textile, cellulosic ethanol or juice industries. As this is the first report of launching leaf enzyme production platform, a complete cost analysis is not yet available. However, the fact that PhyloZyme has attracted investment from owners of textile or microbial enzyme industries underscores the need for a cost-competitive new platform technology. Most importantly, this new platform offers alternate use for tobacco. More than 90% of tobacco is grown in low- and middle-income countries (LMIC — 40% in China, followed by Brazil, Argentina, Bangladesh, Malawi, Zimbabwe); >50% of smokers live in South-East Asia or Western Pacific region. Tobacco kills more than half of its users, more than any other infectious disease (6 million deaths annually, WHO 2017 report). Therefore, producing high-value products (enzymes) will offer an alternate use for this crop, protecting the jobs of LMIC farmers and saving millions of lives.

Materials and methods

Marker-free chloroplast vectors

Daniell laboratory lettuce chloroplast transformation vector, pLSLF (Daniell et al., 2016a,b) containing the aminoglycoside 3'-adenylytransferase gene (aadA), was used as the backbone. To facilitate the aadA gene excision, a fragment of DNA sequence (atpB and 5’ UTR, 649 bp) (Kode et al., 2006) was PCR-amplified using lettuce total genomic DNA as a template, and then, the direct repeats were subcloned into this flanking aadA expression cassette. For insertion of single-digested atpB fragments into the vector backbone, NEBuilder HiFi DNA (NEB, Ipswich, MA) assembly kit was used to avoid the possible ligation of the fragments in the reverse direction. To create pLS-MFPeB, and pLS-MFpeD vectors, the pLD vectors containing the pectinase genes peB and peD (Verma et al., 2010) were digested with NdeI and XbaI and the released fragments containing peB and peD were ligated into the pLSLF-MF vector between NdeI and XbaI sites to replace the ptxD gene.

Selection of transplastomic lines

The marker-free expression vectors, pLS-MFPeB and pLS-MF peD, were bombarded into young (3 weeks old) and fully expanded lettuce leaves through biolistic bombardment as described by Ruhlman et al. (2010). The adaxial side of lettuce leaves was bombarded using 0.6-µm gold particles (Bio-Rad, Hercules, CA) coated with above mentioned vectors, using the bistolic device PDS1000/He (Bio-Rad), 1100 psi rupture discs and a target distance of 6 cm. Following incubation at 25 °C for 2 days, the leaves were cut into small (<1 cm²) pieces and placed adaxial side down on the regeneration media. Primary shoots (4–8 weeks) were analysed by PCR using the three pairs of primers, 16s-F/3M-R, 16s-F/atpB-R and 23s-F/3UTR-R. Leaves from the PCR-positive shoots were again cut into small less 5 mm² pieces and transferred to regeneration medium containing spectinomycin for the second round of selection and then moved to rooting medium, containing spectinomycin (50 µg/mL). Green shoots that on this selection medium were evaluated by PCR for aadA gene excision, and these shoots were transferred to rooting medium without spectinomycin.

Transplastomic tobacco biomass and lyophilization

Tobacco transplastomic lines were selected through germination of seeds on ½ MS medium containing spectinomycin (500 µg/mL), acclimatized in the greenhouse and grown under previously described conditions (Kwon et al., 2018). Hydropotically grown tobacco plants were produced at Fraunhofer CMB (Newark, DE) using previously described conditions (Su et al., 2015), with the following changes: sowing was staggered to increase distance to 4 in² between plants, growth medium comprised 50 ppm Peters Professional 5-11-26 Hydroponic Special and 100 ppm Yara Liva Calcinit 15.5-0-0 and a14/10-h day/night photoperiod. Induced ages of greenhouse-grown plants are from time of transfer from selective media to soil, while hydropotically grown plants are from seed sowing date. Dark green and fully expanded leaves were selectively harvested in the late afternoon/early evening during peak chloroplast protein expression. Leaves were freezedried in a lyophylizer (Genesis 35XL, SP Scientific, Stone Ridge, NY) at −40, −30, −20, −15, −10, −5 and 25 °C for a total of 72 h under a vacuum of 400 mTorr. Lyophilized leaf materials were ground in a coffee mill (Hamilton Beach, Southern Pines, North Carolina) three times at full speed (10 s on and 30 s off). The fine powder was stored with silica gel in containers at room temperature.

Pectinase assay: temperature and pH optima

Pectinase activity in leaf extracts was detected spectrophotometrically at A235 as described previously by Verma et al., 2010. Leaf crude extracts were compared with microbial enzymes in the following commercial products: Pectin Methyl Esterase and Pectin Lyase, Polygalacturonase (Specialty Enzymes and Biotechnologies), Pectinase (BioGreen®), Pectinase 260L (Enzyme Supplies), Bioprep® 3000L (Novozymes®) and Alkaline Pectinase (Sinobios). Enzyme assays included polygalacturonic acid (Sigma-Aldrich, St. Louis, MO) 0.25% as the substrate, Tris-HCl buffer (pH 8.0), Pierre Protease Inhibitor Mini Tablets (ThermoFisher, Carlbad, CA) and sodium azide (0.02%); lyophilized samples were sonicated for 5 s on and 10 s off, 3 times. For pH optimum, the substrate was dissolved in Tris-HCl buffer (50 mM, pH 4.0–11.0) and incubation was performed at 40 °C for 10 min. For temperature optimum, the substrate was dissolved in Tris-HCl buffer (50 mM, pH 8.0), and incubation was performed at temperature (30–90 °C) for 10 min. After mixing completely, absorbance at 235 nm was measured. MilliQ water containing dye and untransformed leaf extract (WT) were used as negative controls. All assays were performed in triplicates.

Fruit juice clarification

For orange fruit juice clarification, the following commercial enzymes were used: Bioprep 3000L; Pectinase 260L, Polygalacturonase (PG) and Pectin Lyase (PL) as compared to the CpPeIA, CpPeIB and CpPeID pectinases. For preparation of leaf pectinases, lyophilized leaves were ground in a coffee mill (Hamilton Beach, Southern Pines, North Carolina) 14 times at full speed (pulse in 10 s each minute). The ground fine powder was stored with silica gel in a humidity-free environment at room temperature. For 200 mg of powder, 2 mL of Tris-HCl (50 mM pH 8.0) was added...
and sonicated for a total time of 25 s; 5 s (ON) and 1 min (OFF). WT untransformed leaf powder served as negative control along with the water blank. To 1 mL of concentrated orange juice, 1 mL of water was added into the 10-mL culture tubes. Then, 400 μL of leaf crude extract and 100 μL purified commercial products were added. Samples were incubated at 50 °C for 120 min with shaking at 150 rpm, pH 5.5. Aliquots of 100 μL were collected at 0, 30, 60, 90 and 120 min, and A700 was measured. To evaluate the stability of the enzyme, activity was measured before and after each assay. After 120 min, the samples were centrifuged for 5 min at 5000 rpm and the amount of galacturonic acid was measured at A235.

**Bioscouring of organic cotton fabric**

The bioscouring of organic cotton fabric was carried out by comparing Bioprep 3000L with chloroplast pectinase (CpPelA). The preparation of the tobacco chloroplast enzymes was performed in the same manner as in the orange fruit juice clarification test. Untransformed plant extract (WT) was used as the negative control along with the water blank. Cotton fabric was cut into similarly shaped pieces and weighed. Each piece was added in a beaker containing water (pH 8.5 or 10.0 maintained using NaOH) along with wetting agent (0.5 gpl). In 25 mL water, 0.1 gpl of enzyme was added and the beaker was placed on a magnetic stirrer with magnetic bar and hot plate was maintained at 60 °C, covered with foil. After 60 min, fabric was rinsed at 80 °C for 10 min. Rinsed fabric was dried at room temperature. Dried fabric was weighed again, and absorption test was performed. To evaluate enzyme stability, activity was measured before and after the assay. The parameters used for analysis were a start point of 10 ms, 25 ms between frames and possible to perform contact angle measurements of water droplets on a solid surface to quantify time and absorption of different fabric materials.

**PAGE and densitometric analysis**

Approximately 100 mg of lyophilized leaf powder was resuspended in 5 volumes of protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl, pH 8, 0.1% [v/v] Triton X-100, 100 mM dithiothreitol, 400 mM sucrose, and 2 mM phenylmethylsulfonyl fluoride) and vortexed vigorously for 20 min at 4 °C prior to determination of total protein using Bio-Rad Protein Assay Reagent. TLPS along with 100 ng of CpPelA, CpPelB and CpPelD tobacco were separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and used for densitometric quantitation. The units of each enzyme were quantified by the amount of galacturonic acid released per min/10 mg leaf powder. For all commercial enzymes, the unit of each enzyme was determined from 1 μL of the commercial enzyme liquid products. For densitometric analysis, ImageJ software was used.

**Statistical analysis**

For temperature and pH optima studies, both chloroplast-derived and commercial enzymes were analysed using three independent biological samples. Enzyme activity assay before or/and after bioscouring and fruit juice clarification experiments were performed in aliquots. For all experiments, mean and standard deviation (SD) values were calculated using Microsoft® Office Excel. The enzyme activity of all commercial products was performed on equivalent enzyme activities, measured under identical conditions because all commercial product labels did not provide enzyme units or weight of non-enzyme filler materials. Therefore, comparison by weight or protein concentration was not feasible.

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**Conflict of interest**

The corresponding author (HD) is an inventor on several patents on expression of enzymes in plant chloroplasts, the technology founder and holds equity in PhylloZyme along with the University of Pennsylvania and investors. He also chairs the PhylloZyme Scientific Advisory Board as a consultant. TR and SL, who generated most of the data presented, do not have any financial conflict. CM is employed by and receives compensation in the form of salary from PhylloZyme. RC and AA received travel support and overhead costs from PhylloZyme and declare financial conflict of interest.

**Author contributions**

TR performed all pectinase enzyme assays in tobacco and lettuce plants and bioscouring and orange juice clarification experiments. PS made marker-free vectors, and SL created and characterized lettuce transplastomic lines expressing PelB and PelD; CM compiled all biomass yield data and pectinase activity in Figure 7; TR, SL and CM contributed to the methods section. RC and AA contributed commercial validation experimental design. HD conceived this project, designed experiments and wrote/edited most of this manuscript except the methods section.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Commercial tobacco leaf pectinase PhylloZyme product.

Figure S2 Commercial edible leaf pectinase PhylloZyme product.

Figure S3 FAMAS software (left) and Contact Angle Meter (right) (Kyowa, Niiza, Japan) used to measure contact angle of water droplets on a surface over time to quantify absorption. Equipment for quantification of absorption by cotton fabric.

Video S1 Cotton fabric treated with water at pH 8.5 and 60°C.

Video S2 Cotton fabric treated with untransformed plant powder at pH 8.5 and 60°C.

Video S3 Cotton fabric treated with Novozymes Bioprep at pH 8.5 and 60°C.

Video S4 Cotton fabric treated with PhylloZymes Pectinase plant powder pH 8.5 and 60°C.