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Abatement of microfibre pollution and detoxification of textile dye – Indigo by engineered plant enzymes

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Summary

Microfibres (diameter <5 mm) and textile dyes released from textile industries are ubiquitous, cause environmental pollution, and harm aquatic flora, fauna, animals and human life. Therefore, enzymatic abatement of microfibre pollution and textile dye detoxification is essential. Microbial enzymes for such application present major challenges of scale and affordability to clean up large scale pollution. Therefore, enzymes required for the biodegradation of microfibres and indigo dye were expressed in transplastomic tobacco plants through chloroplast genetic engineering. Integration of laccase and lignin peroxidase genes into the tobacco chloroplast genomes and homoplasmy was confirmed by Southern blots. Decolorization (up to 86%) of samples containing indigo dye (100 mg/L) was obtained using cp-laccase (0.5% plant enzyme powder). Significant (8-fold) reduction in commercial microbial cellulase cocktail was achieved in pretreated cotton fibre hydrolysis by supplementing cost effective cellulases (endoglucanases, ß-glucosidases) and accessory enzymes (swollenin, xylanase, lipase) and ligninases (laccase lignin peroxidase) expressed in chloroplasts. Microfibre hydrolysis using cocktail of Cp-cellulases and Cp-accessory enzymes along with minimal dose (0.25% and 0.5%) of commercial cellulase blend (Ctec2) showed 88%–89% of sugar release from pretreated cotton and microfibres. Cp-ligninases, Cp-cellulases and Cp-accessory enzymes were stable in freeze dried leaves up to 15 and 36 months respectively at room temperature, when protected from light. Use of plant powder for decolorization or hydrolysis eliminated the need for preservatives, purification or concentration or cold chain. Evidently, abatement of microfibre pollution and textile dye detoxification using Cp-enzymes is a novel and cost-effective approach to prevent their environmental pollution.

Introduction

Microfibres are microscopic threads shed off due to daily activities (diameter <5 mm). They are ubiquitous and found to contaminate aquatic (freshwater and marine), terrestrial and atmospheric environments. Each laundry cycle releases approximately 9 million microfibres into wastewater treatment plants that cannot filter them. At least 13 000–68 000 plastic microfibres from our routine activities of clothing, cleaning carpets, curtains and other textiles per year are released into the atmosphere (Catarino *et al*., 2018). It is proven that microplastics/microfibres in the form of textile fibres enter the waterbodies via washing machines and wastewater treatment effluent. Apparel washing in California was found to generate 2.2 kilotons (kt) of synthetic fibres which shows a 26% increase in the contribution since 2008 and ~80% of this enters the agricultural land in California via biosolid application (Geyer *et al*., 2022). There are some in-drum (GuppyFriend, Cora Ball and Fourth element washing bag) and out-drum (Microplastic Lint LUVR, XFiltra, PlanetCare) devices available which can reduce microfibre release 25%–80% weight into the environment. Nevertheless, these devices enter the environment after their disposal (Belzagui and Gutiérrez-Bouzán, 2022). In addition, household electric dryers are a major source of microfibre pollution, releasing 433 128–561 810 microfibres within 15 min of use (Tao *et al*., 2022), suggesting that electric household dryers generate 40 times more microfibre pollution than washing machines. Microfibres can be generated from both polyester and cotton textiles. These microfibres are modified with different textile dyes and pose a serious threat of recalcitrance for degradation. Indigo dye is an important organic textile dye in the textile, paper, leather and plastic industries with versatile applications. Because indigo is a synthetic dye ($C_{10}H_{10}N_2O_2$), it is highly recalcitrant for degradation. Effluents containing indigo and other dyes from textile industries pose a serious threat of water contamination; this triggers an imbalance in overall aquatic ecosystem food chains (Aki *et al*., 2020).

Denim is the most appealing fashion apparel but generates waste after tailoring and toxic effluents containing indigo dye. Primarily, denim is composed of cotton and polyester or synthetic fibres (viscose, rayon, etc. in different ratios). It was believed that cotton is a natural fibre and gets easily degraded. However, dyed cotton referred to as anthropogenically modified cellulose is recalcitrant and remains in the environment for long time. The chemical processing of these fibres makes it impossible to degrade naturally (Aki *et al*., 2020). Almost 2.16 million tons of waste jeans are generated in Europe annually and 35%–50% of the same is reused or recycled after sorting (Leonas, 2017; Yousef *et al*., 2020). By 2025, the expected release of microfibres is 70 000 tons per year which is equivalent to dumping 400 million polyester T-shirts in the ocean. Overall microfibre pollution has major repercussions, taking a toll on terrestrial and aquatic living beings. Microfibres were found in southern Ontario Lake and

13% of which resembled fibres generated from denim fabrics. Researchers have found that a pair of jeans can release 56 000 microfibres (Athey *et al*., 2020) per washing cycle. Microfibres were found in the arctic sea as well suggesting the atmospheric release from the electric household dryers. It was estimated that arctic sediments had 2000 microfibres per kilogram and 20% of which was identified as denim microfibres. Despite having high concentration of denim fibres in the sediment, researchers reported single denim microfibre in the rainbow smelt gut (Athey *et al*., 2020). This could be because of cellulolytic activity of gut microbiota of fish. Since the denim fibres are anthropogenic cellulose fibres, digestion by fish gut microbiota could be a major reason for the absence or minimal denim fibres in the fish gut (De *et al*., 2015; Li *et al*., 2018; Liu *et al*., 2016). Microfibre release in the atmospheric and aquatic environment has deleterious effects on the ecosystem. Microfibre exposure leads to delayed recovery of lungs for people with pulmonary diseases, lung cancer, chronic asthma and children (Pauly *et al*., 1998). Also being minute in size, microfibres can penetrate placenta which can lead to severe reproductive damage to developing organs and organelles (Acharya *et al*., 2021). This risk is higher in darker denim clothes as compared to light blue denim clothes (Herrero *et al*., 2019).

Because cotton fibre contains 88%–96% cellulose, it is possible to hydrolyse cotton waste by enzymatic or chemical methods to obtain glucose and then ferment it into value-added products. A variety of adsorbents are used to trap indigo dye and other textile dyes including magnesium salts, calcium oxides, rice husk, wheat husk, bagasse, saw dust, charcoal, etc. for dye removal from wastewater but disposal of adsorbents is a significant challenge (Achieng *et al*., 2019; Eletta *et al*., 2018; Gulluce *et al*., 2020). Indigo and other textile dyes can be removed by microfiltration or nanofiltration. However, these methods are expensive (Lebron *et al*., 2021; Murthy and Gowrishankar, 2020). Immobilized Laccase on chitosan beads used to degrade indigo dyes, is not economically feasible (Kumar *et al*., 2022). Ecofriendly degradation of textile dyes is needed, including laccases and peroxidases produced by fungi or bacteria but they are not suitable for addition to industry effluents (Choi, 2021). Ligninases viz; Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) and Lignin Peroxidases (LiP, EC 1.11.1.14 – lignin peroxidase) have been reported to degrade indigo dye in solution as well as fabrics through the possible mechanism of oxidation to isatin followed by its conversion into anthranilic acid (Campos *et al*., 2001). Another mechanism involves aromatic rings of indigo dye and the hydrocyclic groups forming hydrogen bonds with these enzymes including cellulases (viz; endoglucanases and other similar proteins) leading to improve enzyme-substrate binding reactions for degradation of indigo dye in solution and from the denim microfibres (Gusakov *et al*., 2001).

This led us to hypothesize that the denim microfibre from the electric household dryer and the indigo dye can be successfully degraded using a cocktail of cellulases and ligninases expressed in plants (Figure 1). Foreign genes expressed via the nuclear genome has benefited our society for a few decades by reducing the use of pesticides or herbicides. Related to this project, bacterial and fungal ligninases have been expressed via the nuclear genome (Hood *et al*., 2003; Ligaba-Osena *et al*., 2017; Preethi *et al*., 2020; Sonoki *et al*., 2005; Wang *et al*., 2004). Tobacco transient system has been mostly used for the expression of biopharmaceuticals and most recently for producing vaccines against the current pandemic (Phase I – Ward *et al*., 2021; Phase III – Hager *et al*., 2022). Recently, a bacterial laccase has been recently expressed via transient expression in tobacco (Eerde *et al*., 2022). Stable expression via the chloroplast genome has been used to confer agronomic traits, expression of vaccine antigens, biopharmaceuticals, biopolymers or enzymes for textiles, detergents or biofuel applications (Agrawal *et al*., 2011; Bohmert-Tatarev *et al*., 2011; Daniell *et al*., 2016, 2019, 2021; He *et al*., 2021; Kumari *et al*., 2019; Petersen and Bock, 2011; Schmidt *et al*., 2019; Verma *et al*., 2010; Viitanen *et al*., 2004). Chloroplast expression of biopharmaceuticals are advancing to the clinic; angiotensin converting enzyme 2 to prevent SARS-CoV-2 infection and transmission is currently in Phase I/II clinical trials (Daniell *et al*., 2022a,b) and preclinical studies are in progress for oral drug delivery to reduce cost by eliminating cold chain and expensive purification process (Srinivasan *et al*., 2021; Daniell *et al*., 2020: Park *et al*., 2020). Stability of freeze-dried plant cells at ambient temperature for many months/years and their efficacy comparable to purified microbial enzymes without need for cold storage makes this system ideal for various industrial applications of enzymes. We report here the expression of laccase and lignin peroxidase via the chloroplast genome and evaluation of decolorization of indigo dye or hydrolysis of denim or cotton fibres and reduction of microfibre pollution.

Results

Construction of chloroplast transformation vectors with *ligninolytic enzymes* gene sequences

Plastid transformation vectors containing two ligninases gene sequences, *Aspergillus oryzae* laccase (*Ao*Lac) and *Phanerochaete chrysosporium* lignin peroxidase (*Pc*LiP), were created for the expression of these enzymes in tobacco chloroplasts (Figure 2). The 1.67 kb and 1.12 kb full length coding sequences of *Ao*Lac (Figures 2b and S1) and *Pc*LiP (Figures 2b and S2), were amplified following RT-PCR using gene specific forward and reverse primer pairs included with *Nde*I and *Xba*I restriction enzyme sites (Table S1) respectively. The full-length cDNA amplicons from the initial pCR Blunt II Topo vector were sequenced. The entire coding sequences of *Ao*Lac and *Pc*LiP (Figures S1 and S2) that correspond to the GenBank accession number XM_001822813.2 and Y00262.1, respectively, were cut out using *NdeI*/*Xba*I restriction enzyme digestions to subclone into same restriction sites of chloroplast transformation vectors for tobacco (pLD) (Figure 2). The pLD vector included 16S-*trn*I and *trn*A-flanking sequences for homologous recombination into the inverted repeat regions of the chloroplast genome for site specific integration (Figure 2). The *trn*I-flanking region harbouring the origin of replication (oriA) site to assist replication of foreign vectors within chloroplasts. The *aadA* gene was driven by the constitutive rRNA operon promoter with GGAGG ribosome-binding site which confers resistance to spectinomycin during selection. Both the genes, *Ao*Lac and *Pc*LiP, were driven by the *Psb*A promoter in pLD vector and include 5′ UTR and 3′ UTR to achieve high levels of expression and confer transcript stability in homoplasmy transplastomic plants respectively.

Evaluation of site-specific integration of transgenes into the chloroplast genome and generation of homoplasmic lines

Several putative Cp-laccase (*Ao*Lac) and Cp-LiP (*Pc*LiP) transplastomic shoots were regenerated from leaves on the regeneration

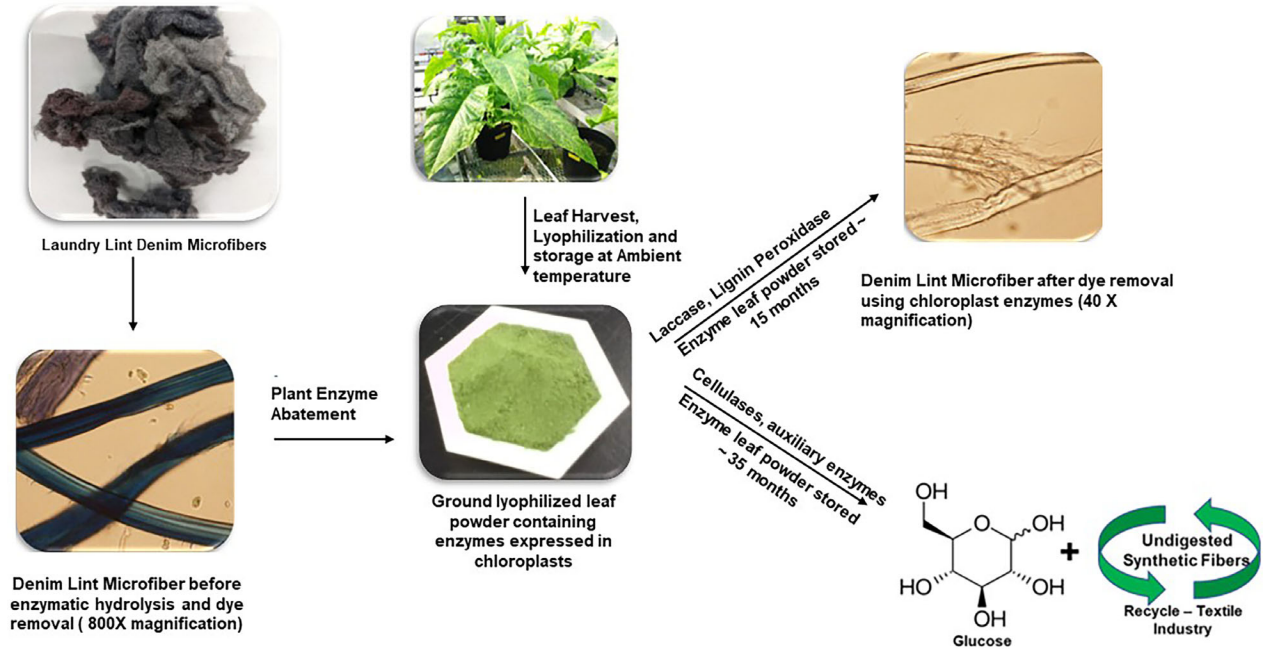

Figure 1 Abatement of microfibre pollution and detoxification of indigo dye by engineered plant enzymes. Laccase, lignin peroxidases, endoglucanase, beta-glucosidase, lipase, xylanase and swollenin were expressed in tobacco chloroplasts and freeze-dried leaf powder was stored for 15, 13, 24, 30, 36, 24 and 35 months respectively, at ambient temperature protected from light in black airtight containers. Enzymatic hydrolysis of microfibre and cotton fibre was conducted using Cp-cellulases and auxiliary enzymes (Cp-xylanase, Cp-lipase and Cp-swollenin) along with minimal amount of commercial cellulase cocktail. Enzymatic degradation of indigo dye in denim microfibre was conducted using Cp-laccases and Cp-LiP at room temperature for 1 h.

medium containing spectinomycin within 3–6 weeks after bombardment with pLD vector coated on gold particles. Primary shoots after 4–8 weeks in second round of selection on spectinomycin plates were transferred on half-strength MS medium containing spectinomycin for the third round of selection to obtained homoplasmic transplastomic plants (Figure 2c). Transplastomic plants were screened for integration of transgene cassettes into the chloroplast genome by PCR using pair of primers given in Table S1 and shown in Figure 2b. PCR analysis using DNA from Cp-LiP transplastomic shoots with *16S rRNA*_F (3P) and *aadA*_R (3 M) primers (Table S1) which anneal with the native *16S rRNA* gene of chloroplast genome upstream to the expression cassettes and the *aadA* gene that located within the gene cassette, respectively, showed the presence of 1.6 kb amplicon and thereby confirmed site-specific integration of transgenes into the tobacco chloroplast genome (Figure 2d). Further presence of *Ao*Lac, and *Pc*Lig, as well as integration of *aadA* genes into chloroplast genome was verified using PCR with *Ppsb*A_F (DV9)/*TpsbA*_R (DV13) and *aadA*_F (5P)/*trnA*_R (2M) primers (Table S1) which anneal at different locations within the transgene cassette as shown in Figure 2b. The presence of around 1.1 kb amplicons in the agarose gel profile of PCR products with DV9/DV13 primers using Cp-LiP transplastomic plants DNA documented precise integration of these genes between *trn*I and *trn*A flanking sequences of chloroplast genome (Figure 2e). Further PCR amplification using aadA_F (5P)/trnA_R (2M) primers documented the presence of around 4.2 kb amplicon in independently developed homoplasmic transplastomic Cp-LiP plants (Figure 2f). Absence of such PCR amplicons in untransformed wild-type (UT-WT) confirmed the site-specific integration into the chloroplast genome of transplastomic plants (Figure 2d–f, lane 2).

We further confirmed the site-specific stable integration of expression cassettes into the chloroplast and homoplasmy status using Southern blot analysis of *Sma*I restriction enzyme digested total plant DNA from transplastomic lines. Unlike a single 4.0 kb hybridization fragment of untransformed wild type (Figure 3, Lane WT) chloroplast genomes, transplastomic Cp-laccase plants showed the presence of ~7.2 kb hybridizing fragment after hybridized with *Bam*HI and *Bgl*II digested flanking *trn*I and *trn*A sequence probe. In the Cp-LiP lines, single 6.7 kb hybridizing fragment was observed, where the absence of a 4.0-kb fragment confirmed the homoplasmy status of the transplastomic lines within the detection levels (Figure 3a,b). Transplastomic lines showed normal phenotype when compared to untransformed plants. Integration of ligninolytic enzyme genes into the tobacco chloroplast genome was confirmed by growing the transplastomic cell lines on 500 mg/L spectinomycin. Seedlings from a homoplasmic transplastomic plants were found to be green on the germination medium plates containing spectinomycin (green, left) and untransformed wild-type (UT-WT, pale white, right) transplastomic cell lines were found to be bleached, showing maternal inheritance of transgenes (Figure 2c).

Characterization of Cp-laccase and Cp-Lignin peroxidase enzymes

Cp-laccase and Cp-LiP plants expressed in transplastomic tobacco plants were grown in the greenhouse and harvested on different days. The harvested samples for Cp-laccase and Cp-LiP were washed, dried, lyophilized and stored at room temperature in black containers protected from light for 15 and 13 months respectively before investigations. The extract of lyophilized ground tobacco plant powder expressing Cp-laccase showed laccase activity of 6000, 9000, 12 000, 14 454 and 17 560 μMol/

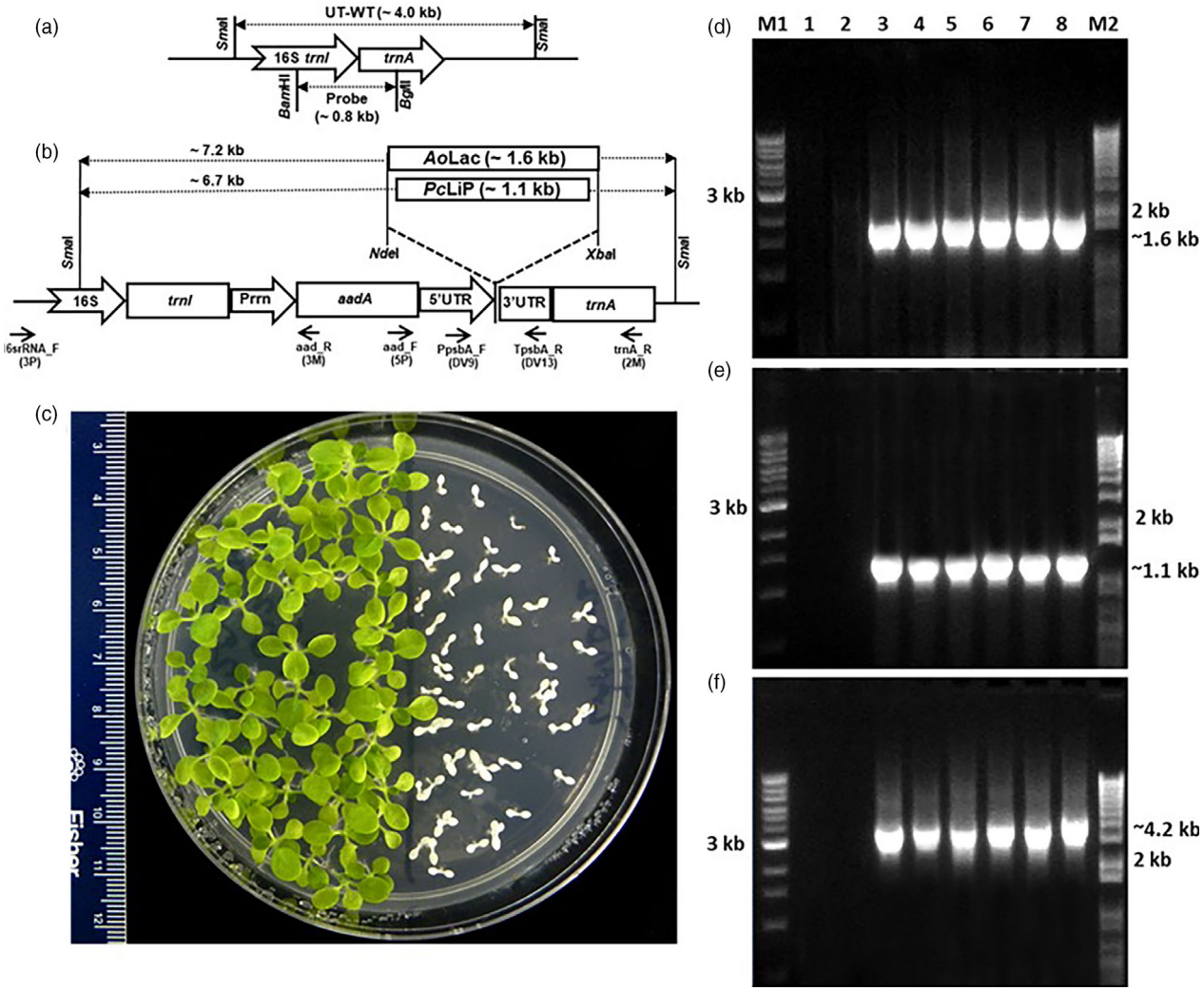

Figure 2 Chloroplast transformation vectors, site-specific transgene integration and maternal inheritance of laccase and lignin peroxidase genes. (a) Schematic representation of chloroplast *trnI*/*trnA* spacer region of untransformed wild-type (UT-WT) tobacco (b) chloroplast vectors of laccase (Lac) and lignin peroxidase (LiP) genes. Solid arrowhead with dotted line represents an expected DNA fragment size of ~4.0 kb from UT-WT, and ~7.2, ~6.7 and ~6.6 kb from *Ao*Lac and *Pc*LiP transplastomic lines after digestion with *Sma*I restriction enzyme. A ~0.8 kb *Bam*HI and *Bgl*II restriction enzyme digested DNA fragment was used as probe for Southern blot analysis. Map is not in scale. (c) Seedlings from a homoplasmic transplastomic line (green, left) and untransformed wild-type (UT-WT, bleached white, right) germinated on 500 mg/L spectinomycin plate showing maternal inheritance. (d–f) Confirmation of integration of ligninase genes into the tobacco chloroplast genome. (d) Amplification of ~1.6 kb fragment using *16SrRNA*_F (3P)/*aadA*_R (3M). Amplification of ~1.1 kb (e) and (f) ~4.2 kb fragments using *PpsbA*_F (DV9)/*TpsbA*_R (DV13) and *aadA*_F (5P)/*trnA*_R (2M) from Cp-LiP (*Pc*LiP) respectively. Lanes M1 and M2 are DNA molecular weight ladders (NEB 1 kb ladder and Invitrogen 1 kb ladder plus, respectively). Lane 1 is negative control. Lane 2 is UT-WT. Lanes 3 to 8 are six independent Cp-LiP transplastomic lines.

h/g at 39, 45, 98, 119 and 126 days respectively by oxidation of 6 mM of ABTS at 420 nm (ε = 36 000 cm^{-1}/M) in 50 mM citrate phosphate buffer (pH 4.5) at ambient temperature (Figure 4a). A 119-day-old sample assessed for Cp-laccase activity at different temperature and pH, showed maximum enzyme activity of 11 500 and 14 700 μMol/h/g at 30 °C and pH-4.5 respectively. Cp-laccase was found to be fairly stable between the temperature range 30–40 °C and pH ranging 4.5–6 (Figure 4c,d). Cp-laccase activity was expressed as μmol of ABTS oxidized per hour per gram of total leaf protein. The wild type showed 2460 μMol/h/g of laccase activity. Assays were evaluated for three biological replicates of the Cp-laccase extracts. The extract of lyophilized ground tobacco plant powder expressing Cp-LiP showed activity of 398, 445, 483, 530 and 615 μMol/h/g using Azure B as a substrate at 49, 56, 62, 70 and 98 days respectively (Figure 4b). Cp-laccase (126-day-old) and Cp-lipase (98-day-old) showed 17 560 ± 1530 and 615 ± 79 μMol/h/g of enzyme activity at <1 month and 17 009 ± 1050 and 589 ± 96 μMol/h/g enzyme activity after 15 months and 13 months of storage at room temperature in airtight container protected from light suggesting no loss of enzyme activity during storage (Figure S3). Cp-LiP activity was expressed μmol of azure B oxidized per hour per gram of total leaf protein. The wild type showed 111 μMol/h/g of laccase activity. Assays were evaluated for three biological replicates of the Cp-LiP extract. Both Cp-laccase and Cp-LiP showed an age-dependent steady increase in enzyme protein

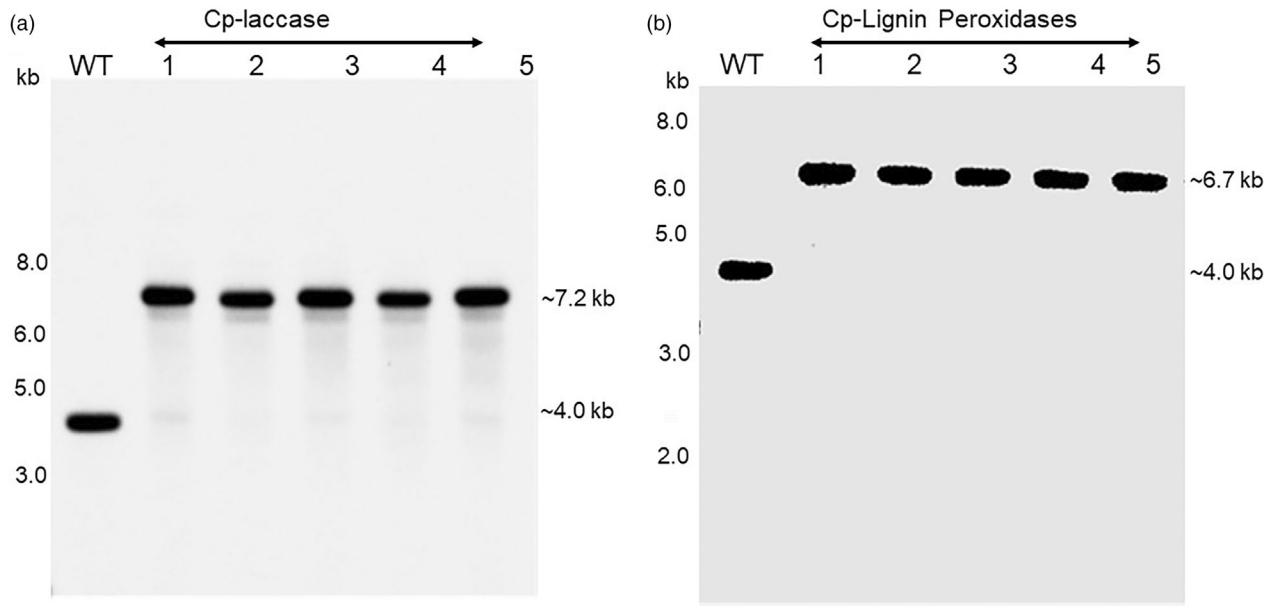

Figure 3 Evaluation of homoplasmy of transplastomic Cp-laccase (*Ao*Lac) and Cp-LiP (*Pc*LiP) plants by Southern blot analyses. Southern blot hybridization profile of *Sma*I restriction enzyme-digested DNA isolated from leaves of Cp-laccase, Cp-LiP transplastomic and untransformed WT plants after hybridization with the probe. Presence of a single ~7.2 kb and ~6.7 kb band in five independent (lanes 1–5) lines of Cp-laccase (a) and Cp-LiP (b) transplastomic plants confirms stable integration and homoplasmic status, whereas existence of a single ~4.0 kb is expected WT (lane WT) plant (see Figure 1a and b for chloroplast vector maps). Membrane after DNA transfer was hybridized with ~0.8 kb *Bam*HI and *Bgl*II restriction enzyme-digested DNA fragment as the probe for Southern blot analysis.

activity. A 70-day-old sample assessed for Cp-LiP activity at different temperature and pH showed maximum activity of 550 and 560 μMol/h/g at 30 °C and pH-4.5 respectively. Cp-LiP was observed to be stable between the temperature 30–40 °C and pH 4.5–6.5 range (Figure 4e,f). High activity samples for Cp-laccase (126 days) and Cp-LiP (98 days) were used in further investigations of indigo dye degradation and microfibre degradation.

Enzymatic degradation of indigo dye using laccases

Indigo dye degradation was evaluated at ambient temperature using crude extracts of 126 days old, lyophilized Cp-laccase powder and 98 days old Cp-LiP powder at 5.0 mg/mL dose for 1 h using 100 mg/L indigo dye concentration in the reaction mixture. Cp-laccase showed 86% degradation of indigo dye whereas Cp-LiP showed 63% degradation. Wild type tobacco powder showed 2% degradation of the indigo dye. Negative controls with inactivated Cp-laccase, Cp-LiP and wild type powder using 1% sodium dodecyl sulphate (SDS) showed 2% indigo dye degradation after 1 h of incubation (Figure 5a,b). The dose–response curve for Cp-laccase at 1.25 mg (6.5 μMol/h/g), 2.5 mg (11 μMol/h/g) and 5.0 mg (22 μMol/h/g) of plant powder per mL of reaction mixture for 1 h, showed 42, 73 and 86% degradation of the indigo dye respectively. The dose–response curve for Cp-LiP for 1 h using 1.25 mg (0.77 μMol/h/g), 2.5 mg (1.54 μMol/h/g) and 5.0 mg (3.08 μMol/h/g) of plant powder per mL of the reaction mixture showed 32, 43 and 63% degradation of indigo dye (Figure 5c). Besides, the high redox potential of Cp-LiP, Cp-laccase was found to decolorize the indigo dye maximally. Cp-laccase and Cp-LiP showed linear dose-dependent degradation of the indigo dye (Figure 5c). However, at equal plant powder concentrations, Cp-laccase showed 36% more degradation of indigo dye as compared to Cp-LiP. Spectral scans of Cp-laccase showed maximum destruction of the peak at 608 nm as compared to Cp-LiP. Spectral scan of wild-type-treated indigo sample showed negligible degradation of dye (Figure 5d).

Enzymatic degradation of indigo dye released from denim microfibres

Enzymatic hydrolysis of lint denim microfibre released blue colour dye in the reaction mixture. Denim microfibres obtained before and after enzymatic hydrolysis were observed under phase contrast microscopy (800×). Denim microfibres were found to be colourless to pale bluish violet after enzymatic dye removal (Figure 6d). The reaction mixture showed maximal absorption peak at 608 nm indicating the presence of the indigo dye after hydrolysis of indigo-dyed cellulose fibres in the microfibre lint. Indigo dye (released from denim microfibre) degradation using equivalent units (22 μMol/h/g) of Cp-laccase and commercial laccase preparation (LMS 200) showed 92% and 74% degradation after 1 h of incubation at ambient temperature. Wild type showed a 20% degradation of dye after 1 h of treatment at the same process conditions. Untreated dye was used as negative control (Figure 6a–c). Commercial laccase preparation turned the indigo dye sample reddish in colour, however the spectral scan showed destruction of the peak at 608 nm, suggesting degradation of indigo dye. A spectral scan of the Cp-laccase-treated indigo dye sample showed decolorization of intense indigo colour to light purple. Spectral scan of Cp-laccase-treated indigo sample showed thorough destruction of the peak at 608 nm, representing 92% degradation of the dye. Cp-laccase showed 24% more degradation of indigo dye as compared to commercial laccase.

Optimization of pretreatment of cotton fibre using cellulases

Cotton fibre is primarily composed of cellulose; however, the crystallinity index of cotton fibre is high as compared to other

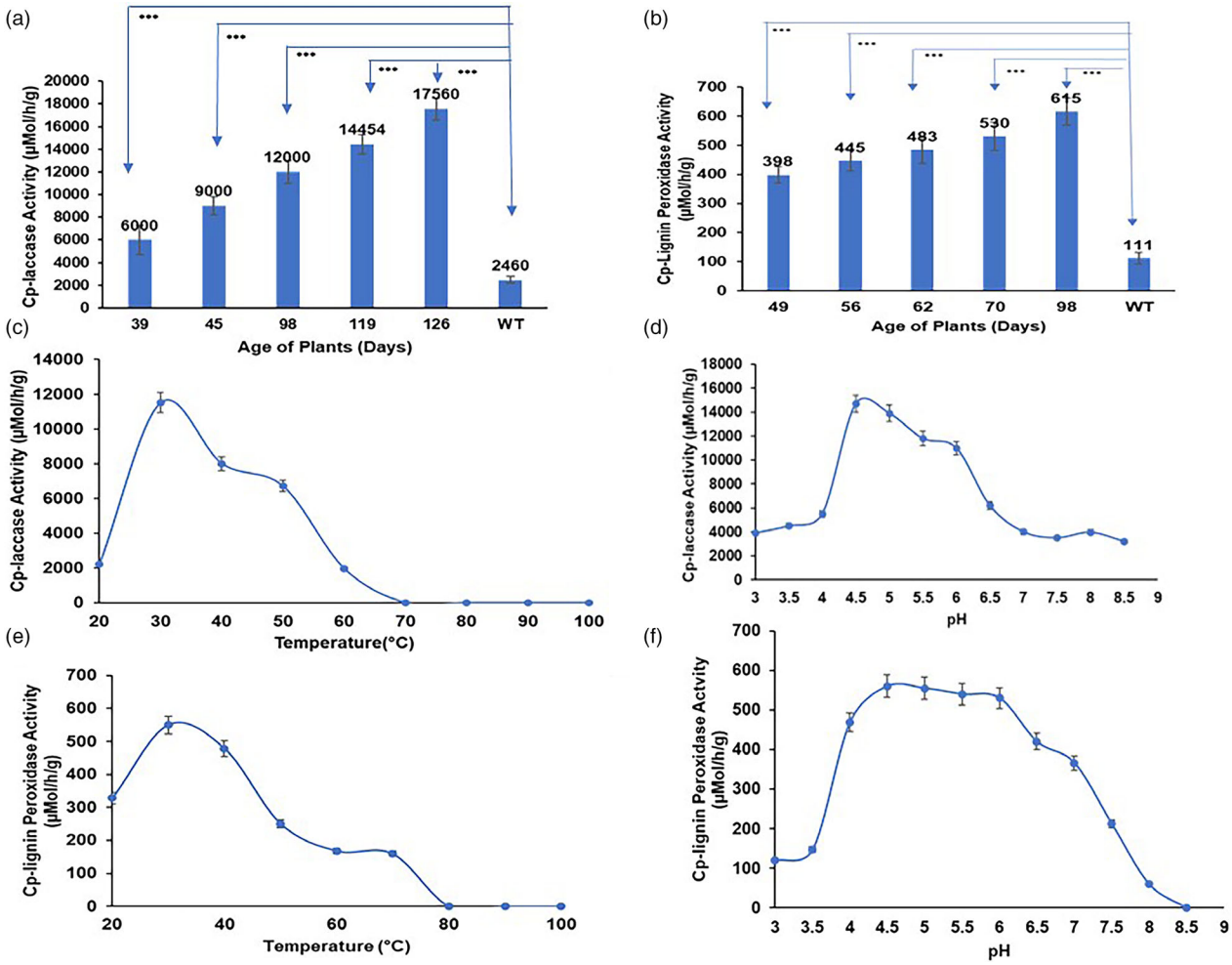

Figure 4 Evaluation of chloroplast laccase and lignin peroxidase activities in untransformed, laccase and lignin peroxidase transplastomic lines: (a) Cp-laccase plants were evaluated and compared with untransformed (WT) plants for enzyme activity. Laccase assay was standardized based on oxidation of micromolar concentrations of ABTS used as the substrate and measured as µmol of ABTS oxidized per hour per gram of total leaf protein. Each plant leaf powder sample (50 mg) was extracted in 1.0 mL of plant extraction buffer (50 mM citrate phosphate buffer pH 4.5) for 1 h at 4 °C on vortex. Plant samples of different ages (39–126 days old) were stored up to 15 months at ambient temperature. The difference in the Cp-laccase activity of different harvests and the wild type is very significant ($P = 0.00171$) analysed by one-way ANOVA. (b) Cp-lignin peroxidase activity of transplastomic plants (49–98 days old) using Azure B as the substrate. Each plant leaf powder sample (50 mg) was extracted in 1.0 mL of plant extraction buffer prepared in 50 mM citrate phosphate buffer pH 4.5 for 1 h at 4 °C on vortex. Assay is based on oxidation of micromolar concentrations of the dye Azure B. Lignin Peroxidase activity is shown as µmol of Azure B oxidized per hour per gram of total leaf protein. Plant samples of different ages (49–98 days old) evaluated were stored up to 13 months. The difference in the Cp-lignin peroxidase activity of different harvests and the wild type is highly significant ($P = 0.00001$) analysed by one-way ANOVA. (c) Effect of temperature on Cp-laccase activity for 119-day-old plant sample stored for 15 months at ambient temperature. (d) Effect of pH on Cp-laccase activity for 119-day-old plant sample stored for 15 months. (e) Effect of temperature on Cp-lignin peroxidase activity for 70-day-old plant sample stored for 13 months at ambient temperature. (f) Effect of pH on Cp-lignin peroxidase activity for 70-day-old plant sample stored for 13 months at ambient temperature.

cellulosic substrates which makes it difficult for enzymatic hydrolysis (Jeihanipour and Taherzadeh, 2009; Zhang and Lynd, 2004). Pretreatment breaks down the hydrogen bonding between cellulose chains reducing its crystallinity index. Pretreatment for cotton was optimized to improve the susceptibility of cotton towards enzymes using commercial cellulase, enzyme blend at 3% dose on cellulose content of cotton (dry basis) and 50 °C for 96 h on shaker for 200 RPM. Hot water, acid and alkali treatments were evaluated at 10% total solid concentration (Figure 7). Hot water and acid pretreatment showed 7 and 8.5% glucose release efficiency respectively. Alkali treatment containing 7% NaOH at 90 °C for 1 h showed 9% of glucose release efficiency. Alkali treatment containing 7% sodium hydroxide (NaOH) with and without 12% urea at −20 °C for 4 h of incubation with intermittent mixing showed glucose release efficiency of 73% and 64% respectively. Negative cotton controls and enzyme controls showed 1% glucose release efficiency suggesting the integrity of polysaccharide with no monomeric sugar release during pretreatment (Figure 7a). Negative enzyme control showed 2% glucose release efficiency which indicates that there is a minimal contribution of monomeric glucose from the enzyme cocktail. Further investigation of

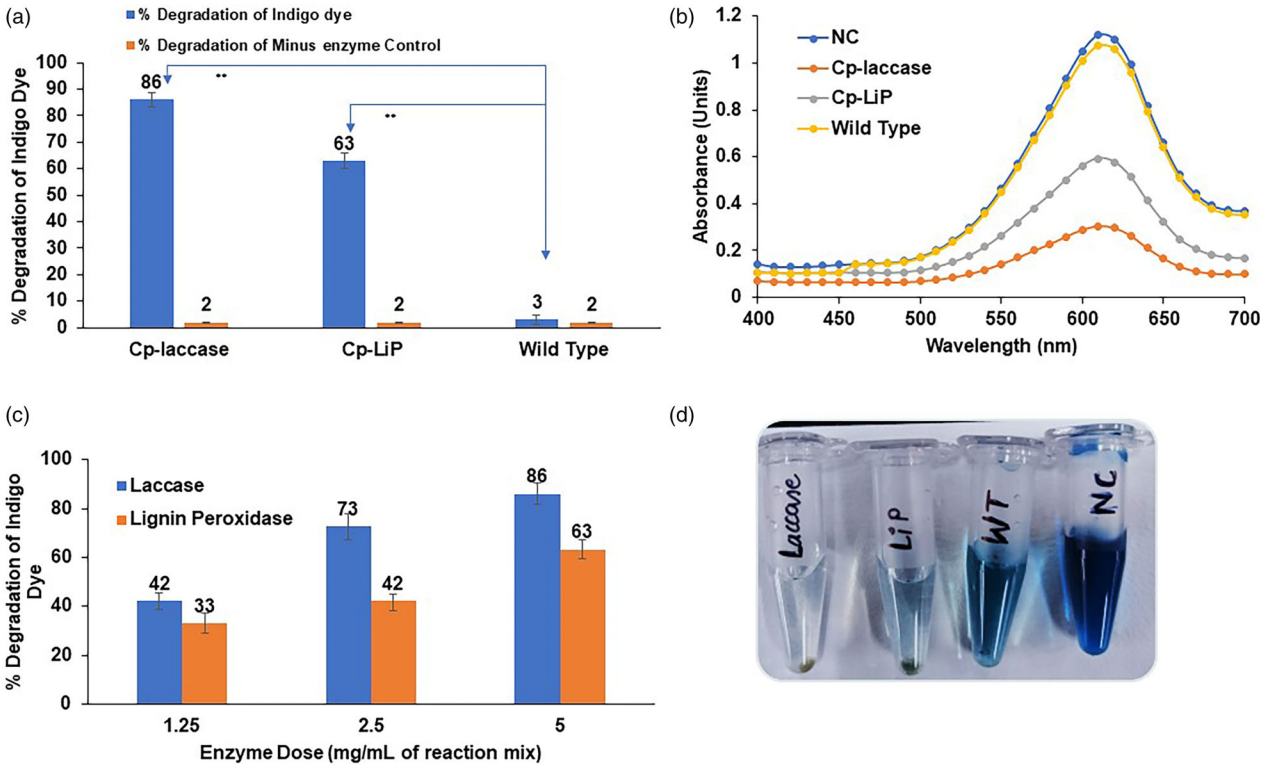

Figure 5 Evaluation of indigo dye degradation using laccase and lignin peroxidase expressed in chloroplasts: (a) Comparative evaluation of Cp-laccase and Cp-lignin peroxidase (Cp-LiP) at 100 ppm initial concentration of indigo carmine dye. Cp-laccase plant powder (126-day old leaf material), Cp-LiP plant powder (98-day old leaf material) and wild type plant (90-day old leaf material) were stored for 15, 13 and 16 months before investigation in black airtight container at room temperature protected from light. Cp-enzyme concentration 5.0 mg/mL was used as the enzyme source. Reaction was conducted at room temperature for 1 h. Copper sulphate was used as cofactor at 20 mM concentration. The difference in the degradation of indigo dye using Cp-laccase and Cp-lignin peroxidase is very significant ($P = 0.02491$) as compared to the wild type analysed by one-way ANOVA. (b) Absorption spectra for Cp-laccase, Cp-LiP, wild type and negative reagent control without plant samples at 100 ppm concentration of indigo carmine dye. (c) Dose-dependent enzymatic degradation of indigo dye using Cp-laccases and Cp-LiP. Cp-laccase using plant powder 1.25 mg (6.5 μMol/h/g), 2.5 mg (11μMol/h) and 5.0 mg (22 μMol/h/g) per mL of reaction mixture for 1 h. Dose–response curve for Cp-LiP for 1 h using 1.25 mg (0.77 μMol/h/g), 2.5 mg (1.54 μMol/h) and 5.0 mg (3.08 μMol/h) of plant powder per mL of reaction mixture. (d) Indigo dye samples obtained after treatment with Cp-laccase, Cp-LiP and wild type plant powder for 1 h shaking (200 rpm) and ambient temperature. NC is negative reagent control without plant enzymes. All experiments were done in triplicate ($n = 3$) and data were expressed as mean ± SD.

enzymatic hydrolysis of cotton and lint microfibres was conducted using alkali pretreatment containing 7% NaOH and 12% urea at −20 °C for 4 h.

Hydrolysis of alkali pretreated cotton fibre using enzyme cocktails

Cp-endoglucanase, Cp-ß-glucosidase, Cp-lipase and Cp-xylanase showed 6502 ± 224, 455 ± 10.2, 7500 ± 305 and 1111 ± 73 μMol/h/g activity at <1 month of the storage and 6196 ± 100, 408 ± 29.1, 7400 ± 238 and 1085 ± 49 μMol/h/g activity on 24, 30, 36, 24 and 35 months respectively suggesting marginal or no loss of enzyme activity (Figure 8c,d). Cp-swollenin was found to show 1.5-fold increased swelling of cotton fibre at room temperature after storage of 35 months protected from light (Figure 8d). Dose–response curve for commercial cellulase blend was studied at different concentration of enzyme viz; 0.5%, 1%, 2%, 3% and 4% on cellulose content for alkali-treated cotton (Figure 7b) at 50 °C for 96 h. Enzymatic hydrolysis of pretreated cotton fibre using 4% commercial cellulase blend showed 99% hydrolysis while 0.5% of commercial cellulase blend showed 19% of glucose release efficiency. For further investigations on optimization of plant enzyme cocktail, 0.5% commercial cellulase blend dose was chosen to obtain 80% sugar unhydrolysed for optimization of Cp-enzymes. Cp-enzymes cocktail containing 1 mg of plant powder for Cp-endoglucanase, Cp-ß glucosidase, Cp-lipase, Cp-Swollenin and Cp-xylanase was prepared and evaluated along with 0.5% commercial cellulase blend at different dose viz; 0.5 mg, 1.0 mg and 2.5 mg concentration. Enzyme cocktail containing 0.5 mg, 1 mg and 2.5 mg plant enzymes along with 0.5% commercial cellulase blend showed 43%, 55% and 88% of glucose release efficiency respectively. The maximum glucose release efficiency of 88% was achieved by synergistic action of different enzymes together. Cp-swollenin and Cp-lipase act as auxiliary enzymes in cotton fibre hydrolysis. Cp-swollenin has a significant role in amorphogenesis of cotton fibre making it susceptible for further hydrolytic action of enzymes (Gourlay *et al.*, 2013; Verma *et al.*, 2013). Similarly, Cp-lipase is important in removing waxy material of cotton fibre and improves its water absorption (Lee *et al.*, 2008). Cp-xylanase helps remove acetyl

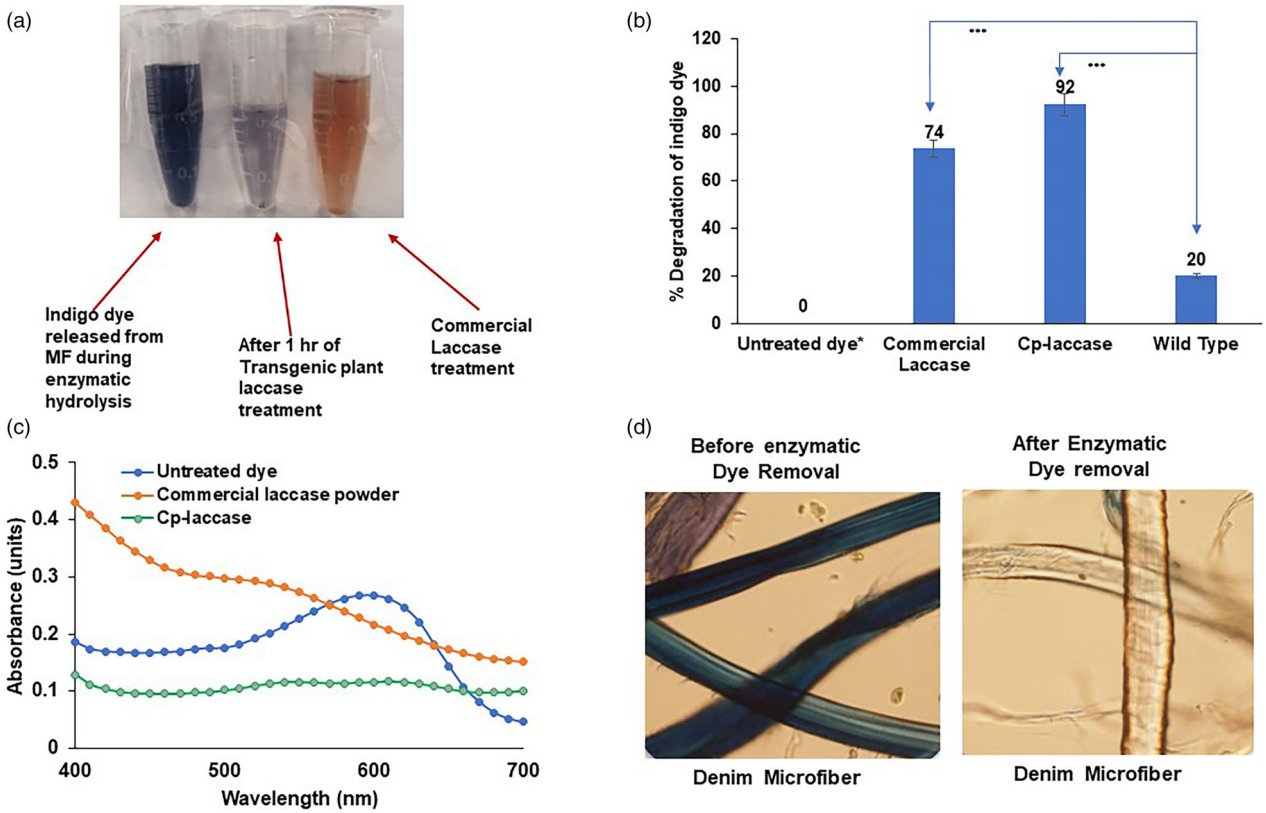

Figure 6 Evaluation of Indigo dye degradation in denim microfibre by plant or commercial microbial laccase: (a) Cp-laccase mediated degradation of indigo dye released after enzymatic hydrolysis of denim lint microfibre. Cp-laccases and commercial laccase preparation were used at equivalent laccase dose of 22 μmol/h/g. A 126-day old Cp-laccase material was used in this investigation, was stored for 15 months in the airtight black container protected from light. The difference in the degradation of indigo dye using Cp-laccase, commercial laccase and the wild type is very significant ($P = 0.0198$) as compared to the wild type analysed by one-way ANOVA. (b) Indigo dye degradation observed after 1 h at room temperature using commercial or Cp–laccase or wild type. For indigo degradation using Cp-laccase and commercial laccase preparation equivalent units of each enzyme 22 μmol/h/g were used. Untreated dye was the liquid collected after denim lint microfibre hydrolysis using Cp-cellulases and Cp-auxiliary enzymes. (c) Absorption spectra for Cp-laccase, commercial laccase treated and untreated indigo dye samples. (d) Denim lint microfibre before and after enzymatic indigo dye removal captured under phase contrast microscope (800× magnification). All experiments were done in triplicate samples ($n = 3$) and data were expressed as mean ± SD.

side chains and expose cellulose for further hydrolysis by cellulases. Negative enzyme control and negative cotton control showed 3% and 1% of glucose release efficiency respectively (Figure 8a). This suggests that addition of Cp-enzymes (enzymes expressed in transplastomic plants) in the cocktail can reduce eight folds dose of expensive commercial cellulase blend. However, minimal dose of commercial enzyme is required because plant enzyme cocktail used in this investigation does not include all enzymes required for biomass hydrolysis.

Hydrolysis of alkali pretreated denim microfibres using enzyme cocktails

The dose–response curve for commercial cellulase blend was studied at different concentrations 0.25%, 0.5%, 1%, 2%, 3% and 4% on solid content of NaOH and urea pretreated denim lint microfibre at 50 °C for 96 h (Figure 7c). Due to unavailability of exact concentration of cellulose in denim microfibre obtained from different variety of denim jeans with respect brand, manufacturer and type of denim fabric (regular, distressed or mildly distressed), enzyme loading was done on solid basis rather than cellulose basis unlike cotton fibre. Optimized cocktail of plant enzymes and commercial cellulase blend was used for denim lint microfibre. The minimum dose of Ctec2 to obtain 20% of sugar release efficiency was found to be two-fold less (0.25%) than that of cotton. This could be attributed to the size, texture and the heat treatment cycle during the drying cycle of the household electric dryer. Selection of the dose was done in such a way that 80% of sugar remains unhydrolysed which can be released through optimization of enzyme cocktail containing different Cp-enzymes mono-components. A cocktail containing 0.25% commercial cellulase blend and 2.5 mg of plant enzymes (0.25 mg of each Cp- Cp-endoglucanase, Cp-ß glucosidase, Cp-lipase, Cp-swollenin and Cp-xylanase) showed 89% glucose release efficiency. This suggests that supplementing Cp-enzymes (enzymes expressed in transplastomic plants), the dose of expensive commercial cellulase blend can be reduced by eight folds. Negative enzyme controls and negative cotton controls showed 2% glucose release efficiency from denim lint microfibre (Figure 8b). Enzyme-treated denim microfibre samples showed intense blue colour after enzymatic hydrolysis (Figure 6a) which was confirmed by its absorption spectrum to be indigo dye and could be released from dyed cellulose fibre after hydrolysis action of enzymes. Light microscopic images of the indigo-dyed

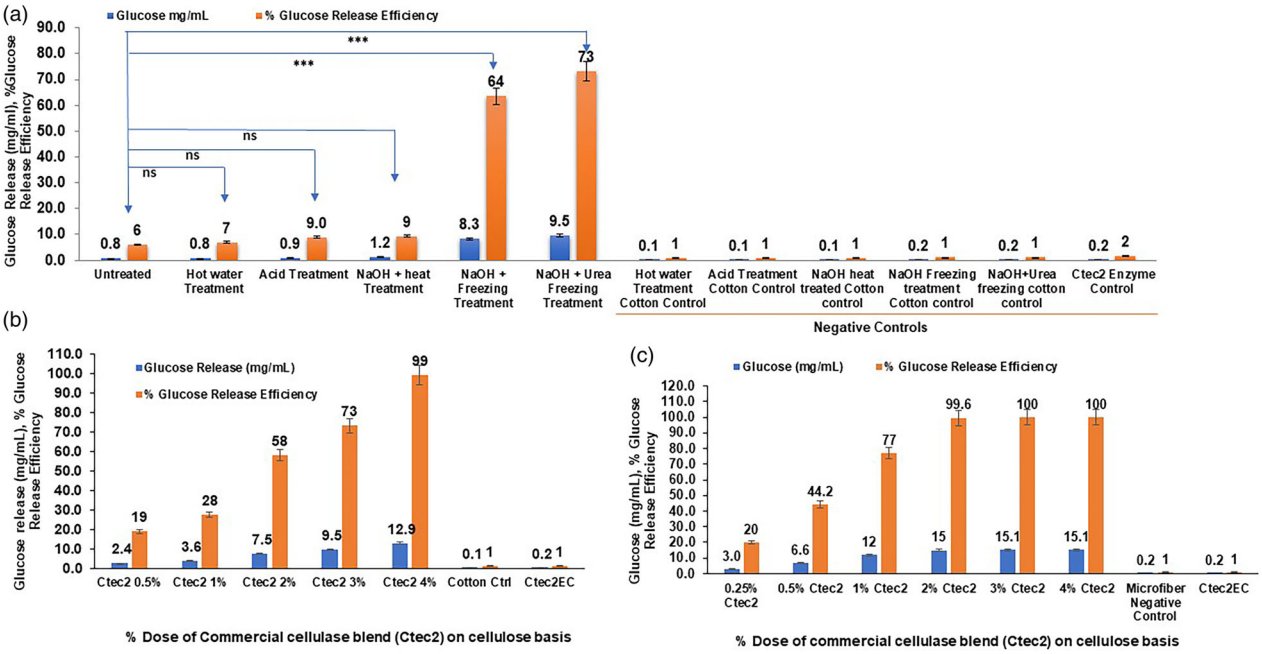

Figure 7 Pretreatment optimization for cotton fibre hydrolysis: (a) Evaluation of different pretreatment conditions for cotton fibre hydrolysis using 3% commercial cellulase blend (Ctec2) and 25 mg cotton (weight of cellulose). Hot water pretreatment was done at 90 °C for 1 h with constant stirring on hot plate. Acid treatment was done using 2% sulphuric acid and 1% acetic acid. Alkali pretreatment was done using 7% Sodium hydroxide (NaOH) at 90 °C for 1 h with constant stirring on hot plate. Sodium hydroxide (7%) + urea 12% treatment at −20 °C for 4 h. For all pretreatments, solid concentration was 10% (cotton fibre). Alkali pretreated cotton fibre substrate using sodium hydroxide with and without urea at frozen conditions, showed highly significant difference ($P = 0.00514$) in the enzymatic hydrolysis efficiency with commercial cellulase Ctec2 blend whereas hot water, acid and alkali pretreatment at elevated temperature did not reveal significant difference as compared to untreated substrate hydrolysis. (b) Evaluation of different doses of commercial cellulase blend (Ctec2) enzyme on 25 mg cotton pretreated with 7% NaOH and 12% urea at −20 °C. Cotton control is negative control without enzymes. Ctec2EC is negative enzyme control without cotton fibre. (c) Evaluation of different doses of commercial cellulase blend (Ctec2) enzyme on pretreated (NaOH and Urea treated at −20 °C, 4 h) denim lint microfibre (DLMF) obtained from household electric dryer on solid basis for 25 mg DLFMFs. Microfibre negative control is negative control without enzymes. Ctec2EC is negative enzyme control without microfibre. All experiments were done in triplicate ($n = 3$). Data were expressed as mean ± SD.

denim fibre after enzymatic hydrolysis were found to be colourless suggesting >80% removal of dye (Figures 1, 6d).

Discussion

Environmental pollution caused by microfibre release and textile dye are growing concerns around the globe. Understanding the gravity of the current situation, we present a possible solution of valorizing the lint microfibre to value-added products and simultaneously detoxifying the textile dye for their safe disposal into the water bodies. Lint microfibres obtained from electric household dryers could be converted to renewable energy through the process of pyrolysis (Yousef *et al.*, 2020). Because this waste is generated in enormous quantities, it is possible to collect it separately if not mixed with other municipal waste. Lint collected from household electric dryer contains approximately 60% of degradable polysaccharides (cellulose) that can be converted to sugars and value-added products by the process of enzymatic hydrolysis. Therefore, hydrolysing denim microfibres from household electric dryer and detoxifying the textile dye using transplastomic plant enzymes could be a practical solution. Unlike microbial enzymes, plant enzymes are stable for several months at ambient temperature without significant loss of enzyme activity over the period of storage. Cp-laccase and Cp-LiP. Cp-endoglucanase, Cp-ßglucosidase, Cp-lipase, Cp-xylanase and Cp-swollenin were found to be stable for 24, 30, 36, 24 and 35 months suggesting the long shelf life at ambient temperature. This alleviates the necessity for cold storage and transportation. Cp-enzymes expressed in field grown tobacco plants can be several orders of magnitude cheaper than microbial/fungal cultivation methods (Daniell *et al.*, 2021; Schmidt *et al.*, 2019).

Detoxification of textile dyes viz; indigo and other dyes have been reported using adsorption, electrochemical treatment, chemisorption, ozonation, microbial biotransformation, membrane filtration, nanocomposite materials, photo catalytic degradation and gamma radiation treatments. These treatments remove toxic dyes efficiently (85%–99% removal efficiency). However, the costs of these treatments limit their usage at industrial scale. On the contrary, Cp-laccase could successfully degrade indigo dye rapidly (1 h) at ambient temperature and low concentration of plant powder without additional physical methods for enzyme release via cell rupture like sonication. This suggests that Cp-laccase plant powder can directly be applied to the effluent containing toxic dyes and dose can be adjusted as per the concentration of dye present in the effluent. In addition, both chloroplast laccase and lignin peroxidase show optimal activity at 30 °C, which is in the temperature of most water bodies (lakes, rivers or industry effluents). The pH range of water in inland fresh waterbodies is 6–8 globally. Particularly, water bodies in Ontario, Canada exhibited pH range 5.77–7.74 (Malyan *et al.*, 2022;

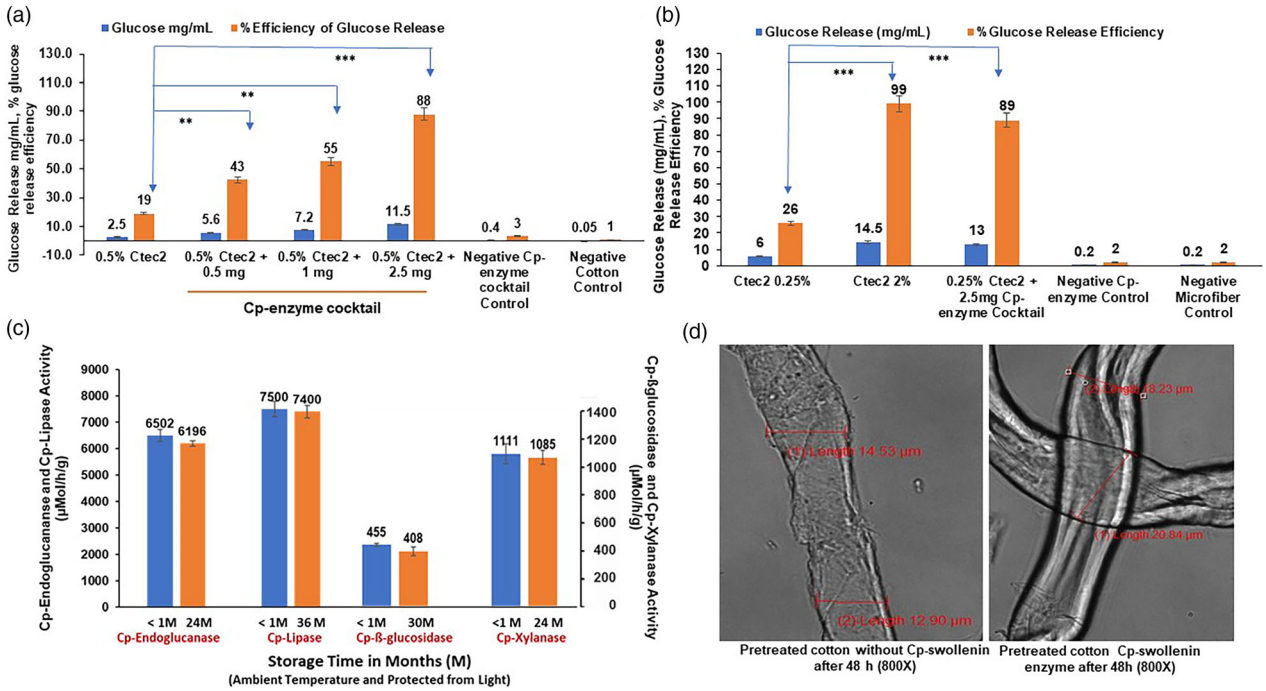

Figure 8 Enzymatic hydrolysis of cotton and denim lint microfibres using microbial and plant enzyme cocktails: (a) Enzymatic hydrolysis of cotton using Cp-enzyme cocktails Cp-endoglucanase (66.5-day-old), Cp-betaglucosidase (94.5-day-old), Cp-lipase (129.5-day-old), Cp-xylanase (63-day-old) and Cp-Swollenin (74-day-old) were stored for 24, 30, 36, 24 and 35 months respectively in black airtight container at room temperature. A cocktail containing 1 mg Cp-Egl + 1 mg Cp-Bgl + 1 mg Cp-lipase +1 mg Cp-xylanase +1 mg Cp-Swollenin, was evaluated at 0.5 mg (2%), 1.0 mg (4%) and 2.5 mg (10%) conc. along with 0.5% Ctec2 (commercial cellulase blend) per 25 mg of cotton. Negative Cp-enzyme cocktail control is negative enzyme control without cotton. Negative cotton control is negative control without enzymes. Enzymatic hydrolysis using Cp-enzymes cocktail at 2.5 mg along with 0.5% Ctec2 showed highly significant difference (P = 0.02827, one-way ANOVA) in the efficiency of enzymatic hydrolysis of pretreated cotton fibre substrate as compared to enzymatic hydrolysis using 0.5% Ctec2 only. (b) Enzymatic Hydrolysis Denim Lint Microfibres (DLMFs) using Cp-enzyme cocktail. Cp-endoglucanase (66.5-day-old), Cp-Betaglucosidase (94.5-day-old), Cp-lipase (129.5-day-old), Cp-xylanase (63-day-old) and Cp-Swollenin (74-day-old) were stored for 24, 30, 36, 24 and 35 months respectively in black airtight container at room temperature. A cocktail containing 1 mg Cp-Egl + 1 mg Cp-Bgl + 1 mg Cp-lipase +1 mg Cp-xylanase +1 mg Cp-Swollenin, was evaluated 2.5 mg (10%) conc. Along with 0.25% Ctec2 (commercial cellulase blend) per 25 mg of cotton. Negative Cp-enzyme cocktail control is negative enzyme control without cotton. Negative microfibre control is negative control without enzymes. Enzymatic hydrolysis using Cp-enzymes cocktail at 2.5 mg along with 0.5% Ctec2 showed highly significant difference (P = 0.0536, one-way ANOVA) in the efficiency of enzymatic hydrolysis of pretreated denim microfibre substrate as compared to enzymatic hydrolysis using 0.25% Ctec2 only. (c) Activity assessment of plant enzymes before and after storage. Cp-endoglucanase (66.5-day-old), Cp-Betaglucosidase (94.5-day-old), Cp-lipase (129.5-day-old), Cp-xylanase (63-day-old) at <1 month and after storage for 24, 30, 36 and 24 months respectively at ambient temperature protected from light. (d) Activity assessment of Cp-swollenin on cotton fibre. Swelling of treated cotton fibre (7% sodium hydroxide and 12% urea treated at −20 °C for 4 h) with and without 1 mg Cp-swollenin at room temperature for 48 h at 200 rpm. Measurement was done using phase contrast microscope (800× magnification). Cp-swollenin (74-day-old) was stored for 35 months at room temperature. All experiments were done in triplicate samples (n = 3) and data were expressed as mean ± SD.

Perron and Pick, 2020). The pH of the denim industry effluent greatly varies from 4.5 to 10 depending upon the fabric and dye used (Hooshmand *et al.*, 2020). Both ligninases show optimal activity in the pH range of 4–7 and therefore could be used. If higher pH is required for alkaline water bodies, bacterial enzymes active in alkaline pH (Eerde *et al.*, 2022) could be engineered via the chloroplast genome, which are indeed more suitable for chloroplast expression.

Similarly, Cp-enzymes used in hydrolysis of pretreated cotton and dryer Lint microfibre waste were applied directly in the form of powder in the reaction mixture. The cocktail containing plant enzymes could hydrolyse 88% and 89% cotton fibre and dryer lint microfibre into glucose with minimal assistance of commercial cellulase blend. The dose of commercial cellulase blend was found to be reduced by eight folds for cotton and lint microfibre. Synergistic action of Cp-auxiliary (swollenin lipase and xylanase) and Cp-cellulases (endoglucanse, ß-glucosidase) could hydrolyse both cotton and lint microfibre at the smallest possible doses (1%) without concentration or purification of enzymes. A minimal dose of commercial enzyme blend was supplemented to initiate the hydrolysis in the first few hours due to the high crystallinity index of cellulose in cotton (Jeihanipour and Taherzadeh, 2009; Zhang and Lynd, 2004) and anthropogenic cellulose present in denim lint microfibres (Aki *et al.*, 2020). Furthermore, the plant enzyme cocktail used does not include all enzymes required for biomass hydrolysis. In lyophilized plant cells, these enzymes are stable for several months or years at room temperature without addition of preservatives. In the developed countries like the United States and other Western countries, electric household dryers are inevitable for drying clothes, which generate huge lint microfibre waste that can be utilized in ecofriendly way of enzymatic hydrolysis using transplastomic

enzymes. Glucose is the principal product of enzymatic microfibre hydrolysis, and this can be used to synthesize value-added biochemicals or bioethanol (biofuel). Thus, the dryer lint can serve as alternative feedstock for the production of biochemicals or bioethanol. Our study reports the feasibility of valorization of dryer lint microfibre to the value-added products and detoxification of indigo dye through the novel approach of using Cp enzymes. For the future commercial applications, Cp enzymes would be evaluated with industrial partners.

Experimental procedure

Ligninolytic enzymes gene sequences and chloroplast transformation vectors

Fungal strains of *Aspergillus oryzae* RIB40 (ATCC 42149) and *Phanerochaete chrysopsorium* VKM F1767 (ATCC 24725) were obtained from the ATCC (Manassas, VA) and cultured on liquid YM medium to harvest hyphal mycelia. Total RNAs were isolated from ground hyphal mycelia powder and full length cDNAs were synthesized using $d(T)_{20}VN$ following the protocol described in Saha *et al*., 2021. Primers for PCR and reverse transcription PCR (RT-PCR) were designed using the PrimerQuest™ Tool (IDT, Coralville, IA; Table S1) and DNA Oligos were synthesized from Sigma (Sigma-Aldrich, St. Louis, MO). A 1.67 kb laccase from *A. oryzae* (*Ao*Lac) and a 1.12 kb ligninase peroxidase from *P. chrysosporium* (*Pc*LiP) gene sequences with respective GenBank accession number XM_001822813.2 and Y00262.1, were amplified following cDNA RT-PCR protocol as described in Saha *et al*. (2021) using gene specific forward and reverse primer pairs included with *Nde*I and *Xba*I restriction enzyme sites respectively (Table S1). RT-PCR amplicons were initially cloned into pCR Blunt II Topo vector (Invitrogen, Waltham, MA) and sequenced from DNA Sequencing Facility (University of Pennsylvania). Sequences were analysed using DNASTAR Lasergene (DNASTAR, Madison, WI) software and NEBcutter V2.0 (New England Biolabs, Ipswich, MA) for internal restriction enzyme sites. Positive clone was digested with *Nde*I and *Xba*I restriction enzymes (NEB) and inserted into same restriction sites of chloroplast transformation vectors for tobacco (pLD) according to Daniell *et al*., 2019.

Tobacco plastid transformation and generation of transplastomic lines

Transformation of the chloroplast genome of *Nicotiana tabacum* cultivar Petite Havana (*Nt*-PH) using pLD vectors were essentially followed the protocol described by Verma *et al*., 2008. Wild-type (WT) surface sterilized seeds were grown aseptically on half-strength MS (Murashige and Skoog, 1962) medium supplemented with 30 g/L sucrose and solidified with 6 g/L agar. Particle bombardment-mediated transformation of chloroplast genome was conducted as described previously (Daniell *et al*., 2019, 2020, 2021) with 0.6-μm gold particles (Bio-Rad, Hercules, CA) coated with pLD vector containing *Ao*Lac and *Pc*LiP gene sequences on the adaxial side of sterile young leaves using the biolistic device PDS1000/He (Bermaio-Rad), 1100 psi rupture discs and a target distance of 6 cm. After incubation at 25 °C in the dark for 2 days, the leaves were cut into small (<1 cm^2) pieces and placed adaxial side down on the regeneration media. Leaves with PCR positive shoots from the first round of selection on regeneration medium of plants (RMOP) on plates containing spectinomycin were further transferred on the same selection medium for another round of selection and subsequently kept on MSO (MS salts without vitamins and growth hormones) medium containing spectinomycin for another round of selection to generate homoplasmic lines. Confirmed transplastomic lines expressing *Ao*Lac and *Pc*LiP were named Cp-laccase and Cp-LiP respectively and transferred to pots in the greenhouse for further growth until maturity.

Molecular characterization of transplastomic lines

Total plant cellular DNA from transplastomic and untransformed WT plants were isolated following a cetyltrimethylammounium bromide (CTAB) extraction method as reported earlier. PCR was conducted following the Verma *et al*. (2008) using a set of forward and reverse primers as presented in Figure 1a and listed in Table S1 for the presence of transgene expression cassettes and site-specific integration into the tobacco chloroplast genome. For Southern-blot analysis, 5 μg total genomic DNA was digested with *Sma*I restriction enzyme (NEB) and followed by size fractionated on a 0.8% agarose gel, and then transferred on to a Nytran blotting membrane (Cytiva, Marlborough, MA) according to standard laboratory protocols. The pUC-Ct vector plasmid DNA was digested with *Bam*HI and *Bgl*II restriction enzymes (NEB) to obtain 0.81 kb chloroplast flanking sequence probe as shown in Figure 1a and described in the Verma *et al*. (2008). Probe was labelled using DIG-High Prime labeling kit (Roche, Indianapolis, IN) and after hybridization with the membrane, the signal was detected using a chemiluminescent CSPD ready-to-use kit (Roche).

Evaluation of maternal inheritance of transplastomic lines

Surface sterilized seeds of WT and independent transplastomic lines were geminated on half-strength MS medium containing spectinomycin for segregation analysis. Aseptically grown plants from culture room were initially transferred to 10-cm pots and kept in the growth chamber for 2 weeks under high humidity, and then moved to the greenhouse at 25 °C for further growth until maturity. Plants were fertilized and irrigated according to standard procedures. Seeds collected from the first-generation plants were germinated on the spectinomycin selection medium (500 μg/mL) for evaluation of their mode of inheritance (Mendelian or maternal).

Characterization of Cp-laccase and Cp-Lignin peroxidase

Cp-laccase and Cp-LiP plants were grown in the greenhouse. Dark green coloured, fully developed transplastomic leaves were harvested in the late afternoon on different days (39, 45, 98, 119 and 126 for Cp-laccase and 49, 56, 62, 70 and 98 days for Cp-LiP) and lyophilized in the lyophilizer (Genesis 35XL, SP Scientific, Stone Ridge, NY) according to the protocol (Daniell *et al*., 2019) for 72 h and vacuum 400 mTorr. Lyophilized Cp-laccase and Cp-LiP samples were ground 10 g each in Biolomix blender for 3 cycles of 12 s. Ground samples were hand sieved through 25-mesh (710 μm) and stored in the Uline black box container at room temperature to avoid exposure to light. The ground samples of Cp-laccase and Cp-LiP were assessed for enzyme activities using 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Wolfenden and Willson, 1982) and Azure B (Archibald, 1992) respectively. Cp-laccase plant powder (50 mg) was extracted in 1.0 mL of 0.05 M citrate phosphate buffer for 1 h at 4 °C on a vortex mixer. After incubation, the sample was diluted appropriately (1:5, 1:10, 1:15, etc.) and laccase activity was measured as a function of time. Laccase Assay is based on oxidation of micromolar concentrations of ABTS and

expressed as μmol of ABTS oxidized per hour per gram of total leaf protein. Similarly, 50 mg of Cp-LiP was extracted in 1.0 mL of plant extraction buffer prepared in 50 mM citrate phosphate buffer pH 4.5 for 1 h at 4 °C on vortex. Cp-laccase and Cp-LiP assays were done at different temperatures viz; 0, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C and different pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5,7.0,7.5, 8.0 and 8.5 to evaluate optimum temperature and pH for maximum enzyme activity respectively. Total leaf protein content for each Cp-laccase and Cp-LiP samples was measured by Bradford assay.

Enzymatic degradation of indigo dye using Cp-laccase and Cp-Lignin peroxidase

Enzymatic degradation reactions were set up in Eppendorf tubes containing indigo carmine dye at 100 mg /L concentration and 5.0 mg of Cp-laccase, Cp-LiP and wild type. Copper sulphate was added as the cofactor of Cp-laccase at 20 mM final concentration. Negative reagent control was prepared without the addition of plant powder. SDS-negative enzyme controls of Cp-laccase, Cp-LiP and wild type samples were prepared by addition of 50 μL of 10% sodium dodecyl sulphate solution to inactivate the enzyme. The reaction mixture was incubated at 4 °C for 1 h on vortex for the enzyme release followed by incubation at ambient temperature on a rotating shaker for enzymatic reaction. Reaction was terminated by placing the tubes in ice bath for 30 min. After termination of the reaction, samples were centrifuged at 18 407 ***g*** for 10 min to remove suspended particles. Samples were read at 608 nm for presence of indigo dye. Degradation of indigo dye was calculated by the formula –

$$\%\text{Degradation of IndigoDye} = \frac{A_{608}\text{Initial} - A_{608}\text{Final}}{A_{608}\text{Initial}} \times 100$$

Enzymatic degradation of indigo dye released from denim microfibre

Enzymatic hydrolysate of 25 mg of denim microfibre using the enzyme cocktail of transplastomic plant enzymes and commercial cellulase blend was collected after centrifugation of denim microfibre hydrolysis reaction tubes at 18 407 ***g*** rpm for 10 min. The supernatant containing indigo dye was carefully transferred to different Eppendorf tube. The supernatant sample was studied for spectral scan 300–700 nm wavelength to confirm the presence of indigo dye. Cp-laccase and commercial laccase blend were loaded at equal dose of 0.22 μMol/h in 1.0 mL of indigo dye supernatant obtained after centrifugation. Copper sulphate was added as the cofactor of Cp-laccase at 20 mM final concentration. The reaction mixture was incubated at 4 °C for 1 h on vortex for enzyme release followed by incubation at ambient temperature on the rotating shaker for enzymatic reaction. Reaction was terminated by placing the tubes in an ice bath for 30 min. After termination of the reaction, samples were centrifuged at 18 407 ***g*** for 10 min to remove suspended particles. Samples were read at 608 nm for presence of indigo dye. Degradation of indigo dye was calculated by the formula as explained in the previous section.

Optimization of pretreatment of cotton fibre using cellulases

Optimization of pretreatment was done on cotton fibre as the substrate. Cotton balls were purchased from local pharmacy store. Hot water and acid (2% sulphuric acid +1% acetic acid) and alkali (7% NaOH) pretreatments were conducted at 90 °C for 1 h with constant stirring on the hot plate using 10% cotton solids as a substrate. After pretreatment, cotton solids were washed with milli-Q water until pH 5.0 using Mira cloth and kept at 37 °C incubator for overnight drying. Similarly, alkali pretreatment was evaluated at frozen conditions –20 °C for 4 h with intermittent stirring. The solid concentration for the pretreatment was 10%. After pretreatment, cotton solids were washed using deionized water until pH 5.0 using Mira cloth and kept at 37 °C for overnight drying. After overnight drying, cotton solids were sieved through 1 mm pore size and 25 mg of the treated substrate (hot water, acid and alkali) was subject to enzymatic hydrolysis.

Pretreatment of denim lint microfibres (DLMFs)

Denim lint microfibres (DLMFs) were collected after heavy heat drying load from 20 different denim jeans having approximate composition 60%–80% cellulose and 20%–40% of synthetic fibres viz: viscose, rayon, polyamide, etc. from the household electric dryer. Dust particles were removed from the DLMFs using a 1-mm pore size sieve. Broken hairs were removed using the forceps. After sorting the dust particles and broken hairs, DLMs were pretreated at the concentration of 10% total solids using 7% NaOH and 12% urea at –20 °C for 4 h with intermittent stirring. After pretreatment, DLMF solids were washed using deionized water until pH 5.0 using Mira cloth and kept at 37 °C for overnight drying. After overnight drying, DLMFs solids were sieved through 1 mm pore size and 25 mg of the treated substrate DLMs were subject to enzymatic hydrolysis.

Hydrolysis of pretreated cotton and denim lint microfibres (DLMFs) using enzyme cocktails

Activity in selected batches of Cp-cellulases (Cp-endoglucanase and Cp-ß-glucosidase), and Cp-accessory enzymes (Cp-swollenin, Cp-lipase and Cp-xylanase) were determined as reported previously (Kumari *et al.*, 2019; Verma *et al.*, 2010). Plant powder for Cp-betaglucosidase, Cp-lipase, Cp-xylanase and Cp-swollenin enzymes were stored for 24, 30, 36, 24 and 35 months before investigations. Crude plant powder extract for all enzymes to be assessed was prepared in 1 mL of respective buffers by suspending 10 mg plant powder and vortexed for 4 °C for 1 h. After incubation, samples were sonicated for 5 s ON and 10s OFF three times on ice bath (homogenates). Homogenates were diluted appropriately before measurement of enzyme activities. Enzyme activity for Cp-endoglucanase was assessed using 2% azo-carboxy methyl cellulose (azo-CMC) at 50 °C, pH 5.5 in 50 mM sodium acetate (Kumari *et al.*, 2019). Cp-ß-glucosidase was assessed using p-nitrophenyl-β-D-glucopyranoside (4 mM) as a substrate at 50 °C, pH 5.2 (Jin *et al.*, 2011). Cp-lipase activity assessment was done using p-nitrophenyl-butyrate 100 mM at pH 8.0 using 100 mM sodium phosphate buffer (Kumari *et al.*, 2019). CP-xylanase was assessed using xylan as a substrate (Leelavathi *et al.*, 2003; Verma *et al.*, 2010) Cp-swollenin was assessed by swelling of pretreated cotton fibre at room temperature for 48 h, 200 rpm (Verma *et al.*, 2013). Plant powder of Cp-swollenin was used at 1 mg concentration for 25 mg of cotton. Negative control without Cp-swollenin was incubated for comparison purpose. Samples were assessed for microscopy and fibre width was measured using a phase contrast microscope (Verma *et al.*, 2013). Enzymatic hydrolysis was performed in Eppendorf tubes containing 2.0 mL of the reaction mixture. Pretreated

substrate (cotton and DLMFs) was weighed accurately in 25 mg amount per reaction tube. A mixture of antibiotics (kanamycin and ampicillin) was added at the final concentration 200 μg/mL to prevent contamination. Dose of Commercial cellulase blend (Ctec2) was optimized for both pretreated cotton and DLMFs to obtain minimal possible release of sugar from the pretreated fibres. Ctec2 enzyme was diluted 1 : 10 with water and loaded at the concentration of 0.5% on cellulose basis. Cp-enzymes viz; Cp-endoglucanase, Cp-ß-glucosidase, Cp-lipase, Cp-xylanase and Cp-Swollenin were weighed in 100 mg amount in a plastic tube of 10 mL capacity. The tube was vortexed for 1 min to get the uniform cocktail of plant enzymes. The plant enzyme cocktail was loaded in the reaction mixture at 0.5 mg, 1 mg and 2.5 mg concentration along with 0.5% commercial cellulase blend. Volume of the final reaction mixture was made to 2.0 mL with Milli-Q water. Negative enzyme control tubes containing respective enzymes were prepared without substrate. Similarly, negative substrate control tubes containing respective substrates (pretreated cotton) were prepared without enzymes. The reaction samples were kept on vortex at 4 °C for 1 h for enzyme release from plant powder. The samples were then transferred to the incubator shaker and incubated at 50 °C for 96 h. Samples were withdrawn and diluted properly to get the absorbance in the range of the glucose standard curve. After dilution samples were centrifuged at 13 523 ***g*** for 10 min to remove undigested cotton/DLMFs and assessed for reducing sugar content using the dinitrosalicylic acid (DNSA) method. Final values reported for all hydrolysis reactions are obtained after subtraction of respective negative substrate controls (Cotton fibre and DLMFs) and negative enzyme controls (Cp-enzymes and Ctec2). Efficiency of enzymatic hydrolysis was calculated based on the maximum glucose release (G_{max}) obtained at the highest enzyme concentrations. Light microscopy of the Cp-laccase-treated samples was done before and after enzymatic dye removal.

Statistical analysis

All enzyme activity assays reported in this investigation were performed in three independent biological replicates and results were expressed as mean ± SD for $n = 3$. For all experiments, Microsoft® Office Excel was used to calculate the mean and the standard deviation (SD) values. Graphpad Prism was used to calculate *P* values for the statistical significance.

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Conflict of interest

Although the corresponding author (HD) is an inventor of several patents on the expression of enzymes in chloroplasts, currently there is no specific financial conflict of interest to report for this project.

Author contributions

HD conceived this project, developed the experimental design for chloroplast vectors, transformation strategies, characterization of transplastomic lines, evaluation of enzymes, analysed/interpreted data and wrote/edited several versions of this manuscript. PS identified and cloned the laccase gene, created the chloroplast vector and wrote corresponding sections. SL bombarded laccase chloroplast vectors and created transplastomic lines. UK confirmed homoplasmy of Cp-laccase and Cp-LiP plants through Southern blots and assays for several plant Cp-enzymes. GW designed, standardized and executed protocols for enzyme assays, pretreatment optimization, enzymatic hydrolysis of microfibres, dye detoxification and wrote corresponding sections of this manuscript.

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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 Coding sequence *Aspergillus oryzae* laccase (*Ao*Lac) gene cloned into the tobacco chloroplast transformation vector (pLD).

Figure S2 Coding sequence of *Phanerochaete chrysopsorium* lignin peroxidase (*Pc*LiP) gene cloned into the tobacco chloroplast transformation vector (pLD).

Figure S3 Enzyme activity of Cp-laccase (126-day-old) and Cp-lignin peroxidase (Cp-LiP) (98-day-old) assessed at 0 month and 15 and 13 months of storage at room temperature in airtight containers protected from light, respectively.

Table S1 Primers used for PCR and RT-PCR analyses.