Discovery of Pectin-degrading Enzymes and Directed Evolution of a Novel Pectate Lyase for Processing Cotton Fabric*

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There is a growing need in the textile industry for more economical and environmentally responsible approaches to improve the scouring process as part of the pretreatment of cotton fabric. Enzymatic methods using pectin-degrading enzymes are potentially valuable candidates in this effort because they could reduce the amount of toxic alkaline chemicals currently used. Using high throughput screening of complex environmental DNA libraries more than 40 novel microbial pectate lyases were discovered, and their enzymatic properties were characterized. Several candidate enzymes were found that possessed pH optima and specific activities on pectic material in cotton fibers compatible with their use in the scouring process. However, none exhibited the desired temperature characteristics. Therefore, a candidate enzyme was selected for evolution. Using Gene Site Saturation Mutagenesis™ technology, 36 single site mutants exhibiting improved thermostolerance were produced. A combinatorial library derived from the 12 best performing single site mutants was then generated by using Gene Reassembly™ technology. Nineteen variants with further improved thermostolerance were produced. These variants were tested for both improved thermostolerance and performance in the bioscouring application. The best performing variant (CO14) contained eight mutations and had a melting temperature 16 °C higher than the wild type enzyme while retaining the same specific activity at 50 °C. Optimal temperature of the evolved enzyme was 70 °C, which is 20 °C higher than the wild type. Scouring results obtained with the evolved enzyme were significantly better than the results obtained with chemical scouring, making it possible to replace the conventional and environmentally harmful chemical scouring process.

Before cotton fabric or yarn can be efficiently dyed it needs to be pretreated to remove materials that inhibit dye binding. The current process used by the textile industry involves three different wet processing steps. During scouring, cotton fabric is treated to solubilize and extract undesired non-cellulosic (pectic) material naturally found in cotton. Scouring uses highly alkaline chemicals to remove the non-cellulosic material; this has a serious environmental impact (1). Additionally, the chemicals partially degrade the cellulose in the cotton fiber, which causes a loss of fiber strength and raw materials; as such, chemical scouring is a non-optimal process.

Mature cotton fiber is composed of a thin primary wall and a thick secondary cell wall. The goal of cotton processing is removal of the primary cell wall. The chemical composition of the fiber is ~95% cellulose and ~5% non-cellulosic. The majority of the non-cellulosic compounds are found in the primary cell wall, which is a complex lattice of pectin (partially methoxylated polygalacturonic acid), protein, cellulose, hemicellulose, and waxes. The secondary cell wall consists almost entirely of cellulose (2, 3). An enzyme that could target specifically the non-cellulosic material could reduce or eliminate the use of harsh chemicals, lessening the environmental burden while maintaining the integrity and strength of the cotton fiber.

Pectinolytic enzymes are primarily produced in nature by saprophytes and plant pathogens (bacteria and fungi) for degradation of plant cell walls. This group of enzymes has been classified into a larger family of carbohydrate-degrading enzymes (4, 5) and can be accessed at the CAZY web site (afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). The nomenclature for the different protein families designated at CAZY is used throughout this report. Fig. 1 illustrates the substrates and products of different enzymes involved in pectin degradation. In brief, pectinases are endo-acting hydrolytic enzymes that digest pectate (non-methoxylated polygalacturonic acid) into oligogalacturonic acids. Exo-polygalacturonidases are exo-acting hydrolytic enzymes that digest pectate, producing galacturonic acid. Pectinases and exo-polygalacturonases together with rhamnogalacturonases form glycoside hydrolyase family 28. Another exo-acting pectinolytic enzyme is exo-polygalacturonase, which belongs to polysaccharide lyase families 2 or 9. Pectin lyases (polysaccharide lyase family 1) are endo-acting enzymes with an eliminative cleavage mechanism on pectin (methoxylated polygalacturonic acid) that produces unsaturated oligogalacturonides. Pectate lyases are endo-acting enzymes that catalyze eliminative cleavage of pectate, producing unsaturated oligogalacturonides. Pectate lyases are found in polysaccharide lyase families 1, 2, 3, 9, and 10. Pectin methyl esterases belong to carbohydrate esterase family 8 and hydrolyze methoxyl groups in pectin, producing pectate and methanol. Another pectin esterase is pectin acetyl esterase, which is present in carbohydrate esterase families 12 and 13. All of these enzymes are involved in the digestion of pectin-related polysaccharides of the plant cell wall.

The source of genes for this study was DNA isolated directly from environmental soil samples. This recovered, highly puri-
Directed Evolution of Pectate Lyase

Optimization of the best wild type enzyme was undertaken by creating sequence variants, screening for improved enzyme characteristics, and then combining the beneficial sequence variations to create further improved variants. Initially, all possible single site mutants of the wild type enzyme were created using the Gene Site Saturation Mutagenesis (GSSM)\(^1\) technology (9). This comprehensive technique introduced point mutations into every position within the target gene, using degenerate primer sets containing 32 codons to generate a complete library of variants. Unlike rational mutagenesis, because of its ability to generate all mutations at all positions within the protein the GSSM technique does not require prior knowledge of the structure or mechanism of the target protein. Unbiased pools of variants are produced for screening and the number of amino acid substitutions is greatly expanded as compared with error-prone and chemical-based mutagenesis. The best performing wild type enzyme was then evolved to deliver superior performance at higher temperatures using directed evolution approaches (7–10, 12–14).

Fig. 1. Enzymes involved in pectin degradation. Arrow 1 activities, pectinase and exo-polygalacturonase. Arrow 2 activities, pectate lyase, exo-polygalacturonate lyase, and pectin lyase. Arrow 3 activity, pectin methyl esterase. For pectate X is predominantly O\(^{-}\), and for pectin X is predominantly O-Me.

Experimental Procedures

Pectinolytic Enzyme Discovery, Expression Screening—More than 2,000 genomic DNA libraries from DNA purified directly from environmental samples, isolates, and primary enrichments have been constructed at Diversa Corporation. Samples were collected under formal agreement with all legal parties. The methods of generating these libraries have been described previously (6, 11). These libraries represent a vast array of ecological niches and biotopes that span the globe. For pectinolytic enzyme discovery, libraries constructed from soil samples containing decaying plant material were targeted. Most of these libraries were constructed from samples from tropical areas like Bermuda, Costa Rica, and Indonesia. Up to 2,000,000 clones/library were plated on semisolid medium according to standard procedures. After plaques had formed, the plates were overlaid with molten substrate (2% azo-rhamnogalacturonan (Megazymes, Bray, Ireland) in 0.7% NZY Top agar). Plates were incubated at 37 °C overnight (~18 h). Clear halos surrounding clones expressing activity against azo-rhamnogalacturonan were detected against the blue background. These clones were purified and excised into phagemids using standard techniques (15, 16).

Sequencing—Plasmid DNA was purified using Qiagen (Valencia, CA) Turbo96 kits on a Beckman (Fullerton, CA) Biomek® 2000 robot according to the manufacturer’s instructions. Sequencing reactions were performed using Applied Biosystems (ABI, Foster City, CA) PRISM® BigDye™ 3.0 terminator kits, using conditions recommended by ABI, and vector primers and internal primers as needed. The DNA sequence was determined using an ABI PRISM® 3700 DNA analyzer. Data were analyzed using Sequencher software from GeneCodes (Ann Arbor, MI).

Annotation and Phylogenetic Analysis of Pectate Lyases—Sequenced DNA fragments encoding pectate lyase activity were annotated using a fully automated annotation pipeline program developed in-house. In brief, open reading frames (ORFs) were identified and then compared with publicly available protein sequences using standard procedures (BLASTP, SignalP, and Hidden Markov Model homology searches). Representative sequences from each pectate lyase family were extracted from the public databases and aligned with the new sequences using ClustalW followed by manual refinement. For phylogenetic analysis, only the pectate lyase catalytic domain of the proteins was used.

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\(^1\) The abbreviations used are: GSSM, Gene Site Saturation Mutagenesis; ORF, open reading frame; PGA, polygalacturonic acid; DSC, differential scanning calorimetry; CBM, carbohydrate binding module; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
because the variable additional domains of the different genes were difficult to align and may not be homologous. Regions in the catalytic domain that could not be reliably aligned were masked from the final alignments. Phylogenetic analysis was performed with the program PROML, which is part of the PHYLIP package, using a maximum likelihood method with Jones-Taylor-Thornton sequence matrix, equal rates, and five random sequence additions followed by global rearrangements. To assess confidence for branching order we used a Bayesian inference approach as implemented in the program MrBayes 3.0. Four Monte Carlo Markov chains were run for 800,000 generations after stabilization of the likelihood values, generating 8,000 trees. A majority rule consensus tree was generated, and the percentage of the time a particular clade occurred, i.e. its posterior probability, was recorded at the major nodes. Values higher than 80–85% were considered a strong support and are equivalent to high confidence values obtained by bootstrap analysis.

Pectate Lyase Activity Assay—Pectate lyase activity was routinely measured using 0.2% (w/v) polygalacturonic acid (Sigma) in 25 mM MOPS (pH 7.2) and 100 mM NaCl to a final concentration of 1 mM. Cells were harvested at 5 h post-induction. The plates were placed in an incubator at 50 °C for 50 min for the GSSM library screen and 70 °C for 25 min for the combinatorial library screen. The plates were resealed and shaken for 10 min. The plates were placed in an incubator at 50 °C for 50 min for the GSSM library screen and 70 °C for 25 min for the combinatorial library screen. The plates were removed from the incubator after undergoing heat challenge and cooled to room temperature. Substrate (180 µl of 0.2% PGA in 25 mM Tris-HCl, 25 mM glycineNaOH, pH 9) was added to each well, and residual enzyme activity was measured by recording absorbance at 235 nm over a 2-min period using SpectraMax instrumentation and SoftmaxPro software (Molecular Devices). For the GSSM library screen, a positive clone was identified as a clone showing pectate lyase activity over background. The primary hits were confirmed by secondary assay (repeat of primary assay) and subjected to DNA sequencing.

RESULTS

Discovered Pectinolytic Enzymes—To target the discovery of pectinolytic enzymes, more than 100 environmental DNA libraries derived from niches rich in plant material (i.e. soil samples rich in decaying plant material) were screened for pectinolytic activity. More than 50 clones were discovered that possessed activity on azo-rhamnogalacturonan substrate. Each positive clone was sequenced, and ORFs were identified and annotated. Bioinformatic analysis revealed that within the ~50 discovered clones there were >70 pectinolytic ORFs, including pectate lyases, pectinas, pectin methyl esterases, and exo-polygalacturonidas. All discovered clones contained either an ORF for a pectate lyase or a pectinase. Additionally, a few clones also encoded an adjacent pectin methylesterase or
exopegalacturonase ORF. Many of the discovered pectate lyases were modular in structure and contained not only a pectate lyase catalytic domain belonging either to polysaccharide lyase families 1 or 10 but also a variety of other modules such as N- or C-terminal carbohydrate binding modules of family 2 and/or 35 (Fig. 2). A few of the pectinolytic enzymes discovered here also contained fibronectin type 3-like modules. These domains are common among bacterial extracellular glycohydrolases; however, their function has not yet been fully elucidated (21). No pectate lyases belonging to polysaccharide lyase families 2, 3, or 9 were found. Several bifunctional enzymes containing a pectin methylesterase domain and a pectate lyase domain from either polysaccharide lyase family 1 or 10 were also discovered. Previous to this study there has been only one report of an enzyme with this structure (22). In some of the polysaccharide lyase family 10 enzymes that were found there were domains that exhibited weak homology to galactose binding domains as well as to an unknown domain. This varied architecture highlights the structural and functional complexity of this group of enzymes. None of the pectinases or the exo-polygalacturonidases discovered here had modular structures (data not shown).

Phylogenetic analysis of the catalytic domains among pectate lyase genes indicates a high degree of sequence diversity. Fig. 3 shows newly discovered sequences, designated by BD prefixes, in relation to other known genes. In general, pectate lyase genes appear to have undergone a series of duplications, as reflected by the presence of multiple paralogues in some bacterial species. It is difficult to predict which bacterial species harbor the discovered genes using the mixed genome approach (10, 11). However, based on sequence similarities, groups of enzymes have been discovered that appear to be closely related to enzymes found in species of Bacillus or Pectobacterium (polysaccharide lyase family 1 enzymes) and in Cellulibrio (polysaccharide lyase family 10 enzymes). A number of newly discovered genes, especially those belonging to family 10, do not seem to have any close relatives among known bacterial species.

Bioscouring Activity of Pectate Lyases—Forty-two purified pectate lyases were subjected to a bioscouring application assay. In each test, an equal amount of enzyme was used for comparative purposes. Six pectate lyases showed substantially better bioscouring efficiency than the rest. Three of these enzymes are members of polysaccharide lyase family 1, and the other three belong to polysaccharide lyase family 10. For both families, the enzymes that exhibited good bioscouring activity also exhibited a degree of sequence similarity (see phylogenetic analysis, Fig. 3). The bioscouring activity of these enzymes was then compared with chemical scouring. Four enzymes (BD8761, BD9903, BD9904, and BD9600) showed equal or better scouring than that obtained with chemical treatment (data not shown). The best performing enzyme (BD9904) was selected for further studies to establish the impact of temperature on bioscouring activity (data not shown). Most efficient bioscouring was obtained at 45 °C. However, 45 °C is likely to be too low for efficient removal of natural waxes from cotton. At higher temperatures performance dropped off dramatically, most likely because of thermal denaturation of the enzyme.

Directed Evolution of BD9904 Using GSSM Technology—Because bioscouring at high temperature would be advantageous for more efficient wax removal, directed evolution methods were used to improve the performance of the lead candidate BD9904 at high temperature. A comprehensive library containing all possible point mutations in BD9904 was constructed using GSSM technology. A thermostolerance screen of the library identified 36 point mutations in 24 different codons that conferred improved thermal tolerance relative to the wild type enzyme. All 36 up-mutant genes were expressed in E. coli. The corresponding enzymes were purified to homogeneity, and their performance in the bioscouring application at 50 °C was tested. At 50 °C, performance of the parental enzyme is decreased relative to its optimum because of thermal inactivation. Most mutants with improved thermostolerance on the surrogate substrate had somewhat similar or reduced performance relative to the wild type enzyme. However, 12 mutants (A118H, A182V, T190L, A197G, S208K, T219M, T223E, S255R, S263K, N275Y, Y309W, and S312V) performed better than the wild type in bioscouring. These mutants were assayed for specific activity and melting temperature. Specific activity measurements (Fig. 4) on PGA at different temperatures revealed that at 30 °C all single site mutants except A182V have lower specific activity than the wild type. However, at 50 °C all mutants except A197G and S208K show improved specific activity over the wild type. The melting temperatures of wild type pectate lyase and the 12 single site mutants were analyzed using DSC. For all mutants, thermally induced denaturation was irreversible, and no discernible transition was observed in a second scan of the sample. The melting temperature (Tm) of the wild type enzyme was 57.3 °C. Mutant A118H had the highest Tm (60.6 °C) melting temperature. No change in Tm was detected for T219M, S263K, and T223E, although they were identified as having improved thermostolerance in the screen. Fig. 5 summarizes the bioscouring application performance and melting temperatures of each of the top 12 single site mutants.

Combination of Single Site Mutations and Identification of Most Improved Variant—It has been noted that the stabilizing effects of point mutations can be cumulative (7, 8, 23, 25). However, some stabilizing single site mutants can provoke negative phenotypic effects, for example, in activity in the application, when present in combination with other up-mutations. A strategy of searching through all possible combinations provides the most efficient route to an optimal mutational combination resulting in the desired phenotypic improvement. To identify variants of the 12 top performing single site mutants with the greatest thermal tolerance and the most improved bioscouring activity, a combinatorial library of all possible mutant combinations (212 combinations) was constructed using Gene Reassembly (10, 13) and screened. Using increased
thermal tolerance as the screening criterion, 19 unique variants containing 5–9 point mutations were identified. Fig. 6 shows a sequence alignment of the unique variants. The alignment shows that A118H and Y309W mutations were found in all combinatorial mutants and S208K, S263K, and N275Y mutations occurred in most of the mutants. These 5 point mutations were present in 9 of 19 variants identified in the thermostolerance screen. Each of the 19 unique variants was expressed in E. coli, and the corresponding enzymes were purified to homogeneity. All purified enzymes were then assayed for their specific activity at various temperatures, and their melting temperatures were determined. Specific activity measurements on PGA at different temperatures revealed that most of the variants, except CO14 and CO22, had significantly lower specific activities at 50 °C than the wild type enzyme (Fig. 7). At 60 °C the wild type enzyme was rapidly deactivated, whereas all variants remained active. Furthermore, at 70 °C three variants (CO14, CO12, and CO8) had much higher specific activities (194, 146, and 143%, respectively) than the wild type enzyme at its optimum temperature.

The melting temperatures of the combinatorial variants ranged from 70.4 to 73.3 °C (Fig. 6), and thermally induced denaturation was irreversible for all variants. The most thermostolerant variant, CO14, had a 16 °C higher melting temperature than the wild type.

All 19 combinatorial variants were subjected to the bioscouring application assay at 70 °C. All variants performed significantly better at 70 °C than the wild type enzyme at its optimum bioscouring temperature (45 °C). The best performer was CO14, which contained eight point mutations (A118H, T190L, A197G, S208K, S263K, N275Y, Y309W, and S312V). Scouring
efficiencies of chemical scouring, bioscouring with the wild type enzyme, the best single site mutant S208K, and the best variant CO14 at their optimal temperatures are shown in Fig. 8.

Both evolved enzymes exhibited activity at a higher temperature than the wild type enzyme and, in addition, showed better bioscouring performance than the wild type enzyme at its optimal scouring temperature.

**DISCUSSION**

The ability to rapidly access and screen DNA derived from discrete biotopes to discover novel enzymes was key to this study; the targeted discovery effort yielded over 50 novel pectinolytic clones. The majority of the clones contained DNA encoding a pectate lyase, although a few encoded a pectinase. Coding sequences for accessory pectinolytic activities, such as pectin methyltransferases and exo-polygalacturonidases, were often found adjacent to pectate lyases or pectinases. Sequence annotation and bioinformatics analyses of discovered pectinolytic clones indicated ORFs that are likely involved in pectin degradation.

A pectate lyase is the pectinolytic enzyme of choice for the bioscouring application for the following reasons. First, pectinases favor acidic conditions for efficient hydrolysis of pectate, whereas pectate lyases have alkaline pH optima that is more compatible with a typical scouring process. Second, under alkaline conditions and elevated temperatures (the optimal conditions for scouring), methyl esters in pectin are rapidly hydrolyzed; therefore, pectic substances in cotton fiber are in the form of pectate, the favored substrate of pectate lyase.

Pectate lyases in the literature are classified into polysaccharide lyase families 1, 2, 3, 9, and 10 (26). In this study, newly discovered pectate lyases were from polysaccharide lyase families 1 and 10. The majority of these enzymes (36 of 42) belonged to family 10, whereas most of the previously known pectate lyase sequences belong to family 1 and only a few sequences are listed for family 10. The original discovery clones encoding the three enzymes from each family that performed best in the bioscouring application test all contained domains (see modular structures, Fig. 2) in addition to the catalytic domain. The additional domains in family 1 enzymes were C-terminal and could not be identified by current bioinformatics analysis tools. Family 10 performing enzymes contained two additional N-terminal domains. The first domain was identified as a family 2 carbohydrate binding module (CBM2) and the second as a family 35 carbohydrate binding module (CBM35). CBM2s are usually ~100 amino acid residues in length (27, 28) and are found in a large number of bacterial enzymes. The cellulose binding function has been demonstrated in many cases. Several of these modules have been shown to also bind chitin or xylan (29, 30). CBM35 comprise a novel family of
The best performer of all variants (CO14) contains the eight side chains (85% identity) is a pectate lyase found in a saprophytic soil due to its location on the surface cleft containing the substrate binding site and catalytic residues, which are located on the top. The side chains are colored red and cyan for wild type (BD9904) and green and yellow for 12×-mutant. The best performer of all variants (CO14) contains the eight side chains shown in yellow. The N-terminal (α/c) toroid subdomain is shown on the right, and the C-terminal mixed α/β subdomain is shown on the left. The surface cleft containing the substrate binding site and catalytic residues is located on the top. The figure was prepared with Molmol (18).

carbohydrate binding modules (31) that is very closely related to CBM6. Interestingly, CBM35s discovered in this study fall into clade 1 in CBM35 in which calcium is required for binding (31). Family 10 enzymes, including BD9904, performed in the bioscouring application only if the N-terminal carbohydrate binding modules were deleted from the expression construct. It may be that the cellulose binding domain and/or carbohydrate binding modules in native family 10 enzymes have affinity for cellulose in cotton fiber and attach the enzyme to cellulose so tightly that, although the enzyme may digest pectin locally, it is not mobile enough to enable complete digestion of pectin, resulting in poor performance. Polysaccharide lyase family 1 enzymes showed performance both with and without the carbohydrate binding domains (data not shown).

Pectate lyases from both families that exhibited bioscouring activity clustered closely together. Three family 1 enzymes (BD9586, BD9600, and BD9862) that share >80% sequence identity over the catalytic domain were found to have the best bioscouring performances among the family 1 enzymes discovered. These enzymes were most closely related (−60% sequence identity) to enzymes found in the plant pathogen Pectobacterium chrysanthemi (γ-proteobacteria). The other three enzymes exhibiting bioscouring activity (BD8761, BD9903, and BD9904) were members of family 10 and also highly similar to each other with ~90% identity over the catalytic domain. The closest relative of these enzymes (85% identity) is a pectate lyase found in a saprophytic soil bacterium Cellvibrio japonicus (γ-proteobacterium). The clustering of enzymes with high activity probably reflects a relatively narrow protein sequence space that is best compatible with the stringency of the conditions in our bioscouring application assay. This highlights the benefits of a functional discovery approach that searches over a wide range of protein sequence space with initial assays that are permissive to most enzymes in a particular family, followed by a targeted assay to identify the best enzymes for a particular application. It is interesting that for the final assay we were able to discover members of both families of polysaccharide lyases that exhibited good bioscouring performance. These enzymes are not homologous, and it has been suggested that they adapted to catalyze the same reaction by convergent evolution (17).

Although it is currently not possible to predict de novo the exact location and nature of amino acid substitutions leading to increased thermostability, the attempt to rationalize the results obtained from the directed laboratory evolution is certainly worthwhile. Previously, several statistically significant, specific amino acid substitutions that occur going from mesophiles to thermophiles have been identified (33), including the increased occurrence of salt bridges in solvent-exposed regions, elimination of destabilizing β-branched residues in helices, and addition of charge-dipole interactions and N-capping boxes in helices. The availability of the three-dimensional crystal structure of the family 10 polysaccharide lyase Pel10Acm from Cellvibrio (17), which has been determined at 1.3 Å resolution and possesses 84% sequence identity to the wild type enzyme, allowed us to investigate the substitutions observed in the laboratory evolution in terms of potential structural changes. Based on the Pel10Acm structure, which is predominantly α-helical and reveals two distinct subdomains separated by a wide surface cleft containing the substrate-binding site and catalytic residues, we calculated homology models for the wild type and for the hypothetical enzyme containing all 12 single site GSSM mutations (Fig. 9). With the sole exception of the single site mutant with the highest melting temperature, A118H, all mutations are located in the C-terminal mixed α/β subdomain. Seven of the 12 single site GSSM mutations could be rationalized in terms of the formation of additional solvent-exposed salt bridges, hydrogen bonds, buried hydrophobic interactions, and helix stabilization by removal of β-branched residues and addition of charge-dipole interactions. The remaining five single site GSSM mutations could not be readily rationalized in terms of structural changes.

Results presented here provide further support (6–14) for the power of the combined use of rapid targeted high throughput discovery and directed laboratory evolution to fully explore and utilize biodiversity and enzyme sequence space (6–14, 19, 24, 32). In this study, biodiversity was explored to discover the best performing pectate lyase in the bioscouring application. The lead wild type enzyme was then evolved in vitro using GSSM technology. This nonstochastic and rapid sampling of all amino acid side chains at every position within a given enzyme led to the discovery of point mutations that introduce functional interactions that may not have been predicted by rational design or captured by traditional mutagenesis methods (e.g. error-prone PCR) that access, at random, one base change per codon. Of the 12 best performing single site GSSM mutants, only one (A197G) was the result of a single base change in a codon; six were double base changes and the remaining five were three-base changes (all positions in the codon were changed). We chose the single site GSSM mutants on the basis of their performance in the bioscouring application and, using Gene Reassembly technology, generated a combinatorial library containing all possible variants of the 12 best performing single site mutants. This enabled us to evolve a novel pectate lyase for significantly better performance at a low enzyme dosage in a high temperature bioscouring process. Enzymatic scouring of cotton fabrics could reduce or eliminate the use of harsh alkaline chemicals, making the scouring process more environmentally responsible while maintaining the integrity and strength of the cotton fiber.

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