In vitro and in silico characterization of metagenomic soil-derived cellulases capable of hydrolyzing oil palm empty fruit bunch

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Diversification of raw material for biofuel production is of interest to both academia and industry. One attractive substrate is a renewable lignocellulosic material such as oil palm (Elaeis guineensis Jacq.) empty fruit bunch (OPEFB), which is a byproduct of the palm oil industry. This study aimed to characterize cellulases active against this substrate. Cellulases with activity against OPEFB were identified from a metagenomic library obtained from DNA extracted from a high-Andean forest ecosystem. Our findings show that the highest cellulolytic activities were obtained at pH and temperature ranges of 4–10 and 30 °C–60 °C, respectively. Due to the heterogeneous character of the system, degradation profiles were fitted to a fractal-like kinetic model, evidencing transport mass transfer limitations. The sequence analysis of the metagenomic library inserts revealed three glycosyl hydrolase families. Finally, molecular docking simulations of the cellulases were carried out corroborating possible exoglucanase and β-glucosidase activity.

1. Introduction

In recent years, there has been an increase in the use, trade, and production of biofuels, due to the need to replace fossil fuels with renewable energy sources. However, biofuel production has generated an ethical debate because the main raw materials can also be basic food crops (e.g., sugarcane, sugar beet and corn). In consequence, the search for alternative sources such as lignocellulosic material from industrial crops (e.g., sugarcane, sugar beet and corn) is currently generating high amounts of material for bioethanol production.

The degradation of cellulose through enzymatic hydrolysis is carried out by three kinds of cellulases: (1) endoglucanases, which can randomly hydrolyze internal glycoside linkages of the amorphous region of cellulose; (2) exoglucanases that progressively attack cellulose molecules at non-reducing ends of the chain, producing cellobiose molecules; and (3) β-glucosidases that hydrolyze cellobiose into glucose. Although there are several sources of commercially available cellulases produced by microorganisms, their effectiveness depends on their affinity for the substrate. It is expected that cellulases highly specific for OPEFB can produce fermentable sugars from this substrate in an optimized manner, making the process of saccharification easier and reducing costs. In consequence, the identification of cellulases capable of degrading OPEFB with high affinity and good reaction rates is a priority to optimize bioethanol production from this material.
Bioprospecting for microorganisms and their metabolic potential is increasingly used as a strategy to identify novel enzymes that may foster the biofuel industry [9,10]. However, the majority of microorganisms available in an environmental sample are unculturable, and therefore their study and assessment of their full biotechnological potential are difficult [11]. Metagenomics, a culture-independent strategy, has been used for discovering products from DNA isolated directly from the environment [11,12]. This approach has led to the discovery and characterization of a wide range of biocatalysts [11–13], which roused interest for the search of novel cellulases for biofuel production [14,15]. Previous metagenomics studies have reported as many as 105 new cellulases, 60 of which were obtained from soil samples [16]. This work reports the identification and characterization of cellulases from a metagenomic library of a high-Andean forest ecosystem, as part of the studies on metagenomics, microbial diversity and bioprospecting done by the GEBIX Center (Colombian Center for Genomics and Bioinformatics of Extreme Environments) in the Colombian National Park “Los Nevados”. The identified enzymes showed activity against OPEFB and are potentially useful in second-generation biofuel production.

2. Materials and methods

2.1. Metagenomic library

The metagenomic library, which consists of 18,432 clones, was constructed using DNA extracted from high-Andean forests soils from the National Natural Park “Los Nevados” [17]. DNA was purified using the Ultra Clean Mega Soil DNA kit (MoBio) and fragments of approximately 30 kb were ligated to the pCC2POS vector (Epigenic) and used to transform Escherichia coli EPI300™, following the manufacturer’s indications (Epigenic). The identification of positive clones for cellulose degradation was done as published [17]. Briefly, clones capable of growing on minimum salt medium (MM; 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.05% KCl, 0.05% KH₂PO₄, 0.15% Triton X-100, pH 8) were used to be cellulases carriers. Then, these colonies were enriched in Luria-Bertani (LB) medium for 2 days and transferred to a solid MM containing carboxymethyl cellulose. To detect cellulose hydrolysis, Congo red staining was used and the presence of a hydrolysis halo surrounding a colony was taken as a positive clone for cellulose degradation.

2.2. Oil palm empty fruit bunch pretreatment

OPEFB was shredded by grinding to a diameter of 5 mm in a hammer mill and pre-hydrolyzed by soaking in 1% (w/v) H₂SO₄ (100 mL for each 5 g of OPEFB) for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h, followed by autoclaving the acid-treated material at 121 °C for 1 h. The lignocellulosic material was washed with deionized water until the pH was close to 6.5, and then dried in an oven at 45 °C for at least 48 h [5]. Prior to each inoculation, the lignocellulosic material was autoclaved with the media.

2.3. Determination of cellulase activity

Cells harboring clones with cellulolytic activity [17], were grown in 2.5 L of MM [18], containing 12.5 μg/mL chloramphenicol, with pretreated OPEFB (1% w/v) as carbon source to the middle of the exponential growth phase (OD₆₀₀ of 0.4), collected by centrifugation for 30 min at 4500 rpm (2,000 × g), and resuspended in 5 mL of buffer (Tris-Cl 50 mM, NaNCl 100 mM, EDTA 1 mM, 0.15% Triton X-100, pH 8). Cell membranes were disrupted using a Beadbeater (Biospec Products, Bartlesville, OK, USA). After centrifugation for 10 min at 13,000 rpm (17,000 × g), supernatants (crude extracts) were used for enzymatic assays.

Enzymatic reactions were performed in buffer (different buffers were used depending on the pH condition required for the reaction) containing OPEFB (2.5% w/v) and crude extract (1.25 mg/mL), and incubating at varying conditions for two hours with agitation (250 rpm). All assays were done in duplicate. After incubation, samples were centrifuged for 1 min at 13,000 rpm (17,000 × g) in order to eliminate OPEFB. The phenol-sulfuric acid assay [6] was used for quantification of sugars: 50 μL of sample were mixed with 30 μL of 5% phenol and 180 μL of 96% sulfuric acid, incubated for 5 min, and the absorbance was measured at 480 nm. The concentration of sugar was determined using a standard curve generated with eight glucose solutions of varying concentrations ranging between 10 and 400 μg/mL.

To determine the effect of metal ion addition, reactions were carried out in the presence of 10 mM each of MgCl₂, CuSO₄, ZnSO₄ and KCl. These reactions were performed in buffer McIlvaine [19] at pH 5.5 and 50 °C.

The pH effect on reaction efficiency was evaluated by performing hydrolysis experiments under different pH conditions using seven different buffers (KCl-HCl for pH 1 and 2.5, McIlvaine for pH 4, 5.5 and 7, Tris-HCl for pH 8.5 and Borax-NaOH for pH 10). The reactions were carried out at 50 °C in the presence or absence of metal ions. Temperature (10 °C to 70 °C) was analyzed at the pH that displayed the highest activity.

2.4. Cellulase kinetics evaluation

Each clone with evidence of cellulase activity was evaluated by obtaining a time profile of fermentable sugar concentration using different initial OPEFB substrate concentrations (5%, 6.25%, and 7.5%) under the specific reaction conditions of metal ions, temperature, and pH for each one. The crude extracts were added when reactions were started. Samples were collected every three minutes up to twenty-seven minutes. Reducing sugars were quantified using the phenol-sulfuric acid method [6]. The experiments were performed by quintuplicate. The results were adjusted to a semi-empirical fractal-like kinetic model (Eq. (1)) [20,21], where [S]₀ is the initial concentration of substrate, kᵣ is a kinetic constant and h is the fractal dimension. The kinetic constant represents the affinity and velocity of the reaction and the fractal dimension represents the influence of the transport phenomena on the reaction kinetics. The fractal dimension value represents the effect of the surrounding factors such as the average diffusion distance of the protein in the 3D space, the 2D diffusion over the cellulase surface and the adsorption rate. The parameter kᵣ is mostly related to the efficiency and affinity of the proteins for the substrate and also the ability of the different kinds of cellulases to interact synergistically. To find the values of each constant, a regression was performed by the least squares method and corroborated by the open fitting curve toolbox (ctool) on Matlab™ (http://www.mathworks.com/products/curvefitting/).

\[ P(t) = [S]₀(1 + \exp(-kᵣt^{(1-h)})) \]  

(1)

2.5. Fosmid DNA extraction and sequencing

Fosmid DNA was purified using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad), according to the manufacturer’s protocol, and resuspended in a final volume of 75 μL. The DNA quality was verified by 2% agarose gel electrophoresis in 1 × TAE buffer. The quantity and quality were analyzed by measuring absorbance 260 nm/280 nm in a Nanodrop™ ND-1000 (Thermo Scientific). The isolated DNA was sequenced by Ion Torrent (314 chip) (Life Technologies, Carlsbad) at the Huck Institutes of Life Sciences, Pennsylvania State University.

2.6. Determination of sequences coding for cellulases

The quality of the reads was checked with the FastQC package [22] and trimmed and filtered by quality using FASTX-ToolKit [23].
Sequences that mapped to the E. coli K12 genome and the pCC2FOS™ sequence (EU140752.1) were removed using Bowtie™ [24]. The de novo assembly of the reads was carried out with CLC™ Genomics Workbench software [25]. Cellulase sequences were identified by running a Basic Local Alignment Search Tool (Blastx) [26] analysis against the non-redundant (nr) protein sequences database (parameters by default). The reads that showed identity over 75% to cellulase sequences were evaluated using Pfam [27] to determine the family to which each hypothetical cellulase belongs.

2.7. Determination of amino acid sequence conservation

Multiple alignments were carried out using ten cellulase sequences (Supplemental Material A1) from other organisms identified using PSI–Blast [26] with three iterations. The alignments were done using MUSCLE [28].

2.8. Structure modeling and molecular docking

The three-dimensional structures of the cellulase sequences were obtained by homology modeling using SWISS-MODEL™ Workspace [29]. The geometric optimization was performed in Hyperchem™ [30], in vacuo using the polak-ribiere algorithm, and RMS gradient of 0.1 kcal/(Å mol) as the termination condition. Each protein model was assessed with the Procheck™ analysis in Swissmodel™ [29,31].

Molecular docking was performed with AutoDock™ 4.2 [32] using a rigid model for the macromolecule. Since the active site was not previously known, the grid was set with a spacing of 0.375 Å and 126 points of evaluation in each direction; thus, the complete surface of the protein was evaluated (blind docking). The genetic algorithm method was used with a number of 10000 evaluations. Fifteen possible binding sites were proposed by AutoDock and those that showed lower binding energy were selected for further analysis.

In order to evaluate exoglucanase capability, all the cellulases were tested with a polysaccharide of five glucose. The identified β-glucosidases were tested with cellobiose. Molecular docking simulations were additionally performed with crystal structures of reference cellulases (gi|327200721 [33], gi|339717359 [34], gi|364506202 [35], gi|16761109 [36], gi|336122540) in order to corroborate the energy magnitude and the location of the substrate. Determination of the amino acids that interact with the ligands was performed with the ‘Receptor-Ligand interaction’ toolbox by Discovery Studio [37].

3. Results

3.1. Identification and kinetic characterization of cellulases

Four fosmid library clones were identified as possible producers of cellulases with activity against OPEFB (clones 4, 8, 12 and 13). These clones were then purified and selected for further characterization. To assay for cellulytic activity, crude extracts were prepared from cultures derived from each clone. The enzymatic activity present in all crude extracts was affected, either enhanced or reduced, by the addition of ions (Fig. 1). All metals, with the exception of Zn+2, enhanced the activity of clone 4 while for clone 8 extract activity was only slightly increased by copper and potassium. In the case of clone 12, the addition of magnesium has a strong inhibitory effect on the degradation in comparison with the other metal additions. For clone 13, which had very low activity in the absence of ions, all metal additions stimulated the hydrolytic activity, being Zn+2 the ion with the strongest effect. Based on these results, potassium was selected for further assays using clones 4 and 8, and Zn+2 for clones 12 and 13.

Reactions were then carried out at different conditions of pH and temperature. The optimum for clone 4 was at the temperature of 50 °C and pH 4 in the presence of KCl, while for clone 8 the best condition was 40 °C and pH 8.5 in the absence of KCl. The activity of clone 12 produced more simple sugars at pH 8.5 and 40 °C, without Zn+2, while clone 13 worked best with this metal at pH 7 and 40 °C.

The time profiles of reducing sugars obtained from hydrolysis were then analyzed by running assays at these optimal conditions of pH, temperature, and metal ion presence or absence. The enzymatic reaction hydrolysis profiles did not adjust to the Michaelis-Menten model because the results showed that the kinetic constant is dependent on the reaction time (Fig. 2).

The observed hydrolytic profiles (Fig. 2) indicated that the increment in substrate concentration had an inverse effect on the fractal dimension (h) and on the quantity of reducing sugars obtained for clones 4, 8, and 13. These values both decreased as the substrate concentration increased; the opposite happened, however, for the h value when clone 12 was analyzed. It is noticeable that the value of k, which could represent factors directly related to the interaction of the molecules, is in all cases in the same order of magnitude.

3.2. Identification of the cellulases via genomic analysis

To identify the possible cellulases present in each clone, each fosmid DNA was purified and sequenced, generating 100,000 reads per clone with a maximum and average lengths of 326 bp and 240 bp, respectively. For each fosmid, approximately 70,000 reads were assembled and the resultant 7000 contigs were analyzed using Blastx [26]. Among the sequences that showed identity with cellulases, two contigs of each clone 4, 8, and 13 and one contig from clone 12 were selected for Pfam characterization. Three glycosyl hydrolase families were found on seven of the contigs (Table 1): β-glucosidases (families 1 and 3), Endoglucanases (family 8) and Exoglucanases (family 3). Contig 131, derived from clone 13, contained a sequence belonging to the peptidase family and it showed a characteristic Zn-dependent domain. This sequence also showed a M42 glucanase-like (cd05657) subdomain that was detected with the Blastx analysis. In summary, the metagenomic inserts from clones 4 and 13 could code for endoglucanase and β-glucosidase cellulases, the insert from clone 8 could produce endoglucanase and exoglucanase cellulases, while clone 12 contained a possible exogluclusase. Based on this analysis it was predicted that amino acid 373 of contig 411, a hypothetical β-glucosidase, could be in the active site of the protein. The analysis of the putative cellulase sequences using the Pfam platform gave low e-values, suggesting that the alignments were not due to randomness. The previously described protein sequences were submitted to UniProt with accession numbers LT853709 to LT853715.

3.3. Structure modeling and molecular docking assays

Further characterization of the cellulases through biochemical
analyses would be possible after extraction and purification of the proteins. However, bioinformatics tools can be used as a first approach to the functionality of the novel cellulases. The proteins’ sequences were modeled by homology based on crystal structures of reported cellulases (reference cellulases) and then geometrically optimized. The quality of the resulting structures was checked with structure assessment (Table 2). More than 96% of the amino acids’ dihedral angles for all structures were in allowed regions. Nevertheless, the 3D structures derived from the sequences of contigs 8II and 13I had a higher percentage of amino acids in disallowed regions of the Ramachandran plot, indicating that some zones should be more carefully refined. The Qmean scores for all structures lied within the range of 0.561 and 0.747. These results indicated that the models had an acceptable quality for simulations.

Molecular docking analyses showed that the endoglucanases

| Contig ID | Qmean6 Score | Residues in allowed regions | Residues in disallowed regions |
|-----------|--------------|-----------------------------|-------------------------------|
| 4I        | 0.659        | 99.6%                       | 0.4%                          |
| 4II       | 0.711        | 99.5%                       | 0.5%                          |
| 8I        | 0.747        | 99.3%                       | 0.7%                          |
| 8II       | 0.561        | 97.4%                       | 2.6%                          |
| 12I       | 0.63         | 99.7%                       | 0.3%                          |
| 13I       | 0.61         | 98.3%                       | 1.7%                          |
| 13II      | 0.63         | 99.8%                       | 0.2%                          |

Table 1
Identification of Pfam domains in each contig that had Blast results related with cellulases.

| Clone | Contig ID | Enzyme expected | Domains                     | Length | Alignment | E-value |
|-------|-----------|-----------------|-----------------------------|--------|-----------|---------|
| 4     | 4I        | Endoglucanase   | Glycosyl hydrolase family 8 | 271    | 2         | 257     |
| 4     | 4II       | β-glucosidase   | Glycosyl hydrolase family 1 | 475    | 4         | 472     |
| 8     | 8I        | Endoglucanase   | Glycosyl hydrolase family 8 | 370    | 3         | 348     |
| 8     | 8II       | Exo-1,3-1,4-glucanase | Glycosyl hydrolase family 3 N-terminal domain | 631 | 25 | 227 | 1.4E-63 |
| 12    | 12I       | Exo-1,3-1,4-glucanase | Glycosyl hydrolase family 3 C-terminal domain | 411 | 51 | 338 | 7.4E-91 |
| 13    | 13I       | Endoglucanase   | M42 glutamyl aminopeptidase | 373    | 46        | 346     |
| 13    | 13II      | β-glucosidase   | Glycosyl hydrolase family 1 | 476    | 4         | 472     |

Table 2
Qmean scores and Ramachandran Plot statistics from 3D modeled structure quality check of each individual contig. The results were obtained on the Swiss Model platform.

Fig. 2. Time profile for the hydrolytic activity of each metagenomic clone using different concentrations of OPEFB (5%, 6.25%, and 7.5%). (A) Clone 4 with the addition of KCl, (B) Clone 8 without any metal ion addition, (C) Clone 12 without any metal ion addition, (D) Clone 13 with the addition of ZnSO4. All the curves were adjusted to a fractal kinetics model (continuous lines). Error bars shown are standard deviation, n = 5.
interacted with a polysaccharide of five glucose and that the β-glucosidases contributed to both ligands, the polysaccharide and cellulose, as expected. These ligand-protein interactions involved conventional hydrogen bonds, carbohydrate-hydrogen bonds, Pi-Sigma and Pi-Donor Bonds, which are located in putative active sites (Supplemental material A3). This analysis showed that most of the residues with cellobiose involved the same homologous sequences. The overall conservation of the amino acids that showed interaction with the ligand was located in the same cavity on 8I and 13I, resulting in cellulases like their respective protein reference. On the contrary, the ligands were located in the same cavity on 8I and 13I, resulting in different. Further analysis revealed that the produced endoglucanase had a motif for recognition of Zn+2, which could be a cofactor for the protein. The temperatures and pH conditions that allowed the best production are within ranges previously reported for other organisms (Supplemental material A3). This analysis showed that most of the residues with ligand interactions in the novel putative cellulases were conserved in the homologous sequences. The overall conservation of the protein sequence was more evident for the proteins derived from 12I and 4I.

### 4. Discussion

In this work, putative cellulases identified in metagenomic clones with enzymatic activity on OPEFB were analyzed. The effect of various metals on enzymatic activity was further evaluated and it was shown that metals can have either a positive or negative effect on hydrolysis. The presence of a metallic element could influence the transport of the cellulase along the fiber as well as the affinity of the enzyme for the substrate. Subsequent bioinformatics analysis of the putative cellulases from clone 13 corroborated the experimental results and showed that the produced endoglucanase had a motif for recognition of Zn+2, which could be a cofactor for the protein. The temperatures and pH conditions that allowed the best production were within ranges previously reported for other cellulases found in similar studies [38–41]. The conditions that we were reporting were the optimal found for the degradation of OPEFB by the crude protein extracts from the selected isolates and therefore could be related to the activity of more than one cellulase present in the fosmid inserts. Currently, there exist commercially available enzymes for production of sugars, such as the ACCELLERASE® (Dupont, Wilmington, DE, USA) product line. The offered products have a wide range of temperatures for activity, from 30 °C to 75 °C, and pH ranges from 3.5 to 7, depending on the product. Although the temperature range of our protein extracts is similar to that of this commercially available product, the optimum pH is higher for the enzymes identified in this study. In consequence, the cellulases available in our extracts could be used in processes where higher pH is needed for specific degradation of OPEFB.

The change with time of the kinetic constant (Hydrolysis profile...
The production of bioethanol from lignocellulosic material has been...
a topic of research and discussion in recent years. Nevertheless, the viability of this process depends on the optimization of the different operations, and in this regard, the identification of cellulases specific for lignocellulosic material could be a significant improvement. In this study, metagenomics was used as a tool to identify novel cellulases with specific cellulolytic activity against OPEFB. Four clones were identified as producers of cellulases active against this substrate. The kinetics of the hydrolysis performed by each clone were adjusted to a semi-empirical fractal-like kinetic model that was previously reported in mass transfer limited reactions.

Three families of cellulases were identified within the resulting contigs of the clones analyzed. Molecular docking allowed the recognition of the active sites and of important amino acids in each cellulase. Through multiple alignments, it was identified that the metagenomics sequences were most probably derived from bacteria. In all cases, the majority of the amino acids that interact with the substrate were highly conserved in homologous sequences, indicating evolutionary preservation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2017.06.003.

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