

Article

Cellulases Production by a *Trichoderma* sp. Using Food Manufacturing Wastes

Felipe Gordillo-Fuenzalida ¹, Alex Echeverría-Vega ² , Sara Cuadros-Orellana ¹,
Claudia Faundez ¹, Thilo Kähne ³ and Rodrigo Morales-Vera ^{1,4,*}

¹ Centro de Biotecnología de los Recursos Naturales (CENBIO), Facultad de Ciencias Agrarias y Forestales, Universidad Católica del Maule, Avda. San Miguel 3605, Talca 3480112, Chile

² Centro de Investigación en Estudios Avanzados del Maule (CIEAM), Vicerrectoría de Investigación y Postgrado, Universidad Católica del Maule, Avda. San Miguel 3605, Talca 3480112, Chile

³ Institute of Experimental Internal Medicine, Otto-von-Guericke University, Magdeburg 39120, Germany

⁴ Facultad de Ingeniería, Ciencias y Tecnología, Universidad Bernardo O'Higgins, Santiago 8370993, Chile

* Correspondence: rmorales@ucm.cl; Tel.: +56-71-2203505

Received: 9 August 2019; Accepted: 16 October 2019; Published: 18 October 2019



Abstract: The cost of cellulase enzymes is a main contributor to the operational cost of a biorefinery producing ethanol from lignocellulosic material. Therefore, onsite production of enzymes using low-value substrates might be an option to make a bio-based facility more economical, while improving environmental sustainability. Food manufacturing wastes (FMWs), such as olive mill solids, tomato pomace, and grape pomace, are some of the main wastes produced by the food industry in Chile. FMWs are mostly composed of lignocellulosic material, which is primarily made of cellulose. A fungal strain obtained from olive stones was identified as a *Trichoderma* sp. and characterized by molecular and morphological techniques. This strain was able to grow on three FMWs in both liquid and solid cultures. In liquid cultures, cellulase and β -glucosidase activities from the culture supernatants were quantified. Identification of extracellular proteins using mass spectrometry revealed the presence of endoglucanases, exoglucanases, and β -glucosidases. Cellulase production from agroindustrial residues could be an excellent opportunity to utilize FMWs as well as decrease enzyme production costs in biorefinery processes.

Keywords: enzymes; food manufacturing wastes; olive mill solid waste; tomato pomace; grape pomace; cellulases; *Trichoderma* sp.; bioethanol; biorefinery

1. Introduction

The production of food manufacturing wastes (FMWs) is potentially damaging to the environment; these materials, if not properly disposed of, can lead to the release of greenhouse gases or contaminate soil and water as they decay. Biotechnological approaches may be one solution to utilize these materials before they cause damage to the environment [1,2].

In Chile, three important waste materials generated from the food manufacturing industry are olive-mill solid waste (OMSW), tomato pomace (TP), and grape pomace (GP). OMSW is a sub-product of olive oil production by two-phase centrifugation. It is composed of a liquid fraction also known as “alpechin” and a semisolid residue known as “alperujo”. The chemical characterization of “alperujo” reveals a high moisture content, slightly acidic pH, and high content of organic matter, mainly consisting of lignin, hemicellulose, and cellulose. Furthermore, “alperujo” contains lipids and water-soluble polyphenols [3,4]. TP is a sub-product of tomato processing which mainly consists of peels and seeds and is composed of lignocellulosic material, proteins, minerals, and lipids [5]. GP is a sub-product of grape processing, mainly composed of lignocellulosic residues, glucan, alcohol, phenols, and flavonoids [6].

One of the simplest strategies for disposal of these FMWs is biodegradation through enzymatic attack. FMWs are mainly composed of lignocellulose, of which cellulose is the major component (20–50% *w/w*) [7]. In nature, a wide range of microorganisms can use cellulose as a carbon and energy source. To accomplish this, fungi and bacteria secrete extracellular enzymes able to degrade this complex polymer [8]. Cellulose is hydrolyzed by three different classes of enzymes: endo- β -1,4-D-glucanases (EC 3.2.1.4), which hydrolyze internal β -1,4-glucosidic linkages, 1,4- β -D-glucan cellobiohydrolases (EC 3.2.1.91), which cleave off cellulose fragments from the cellulose chain ends, generating the cellobiose disaccharide and β -D-glucosidases (EC 3.2.1.21), which use cellobiose hydrolysis to generate glucose [9].

Cellulases have been applied in many different industries, such as food, textiles, pulp, paper and wastewater treatment of residue effluents [10,11]. However, one of the most significant current applications is in bioethanol production from lignocellulosic materials. Bioethanol is considered a fuel alternative for reducing the dependence on crude oil [7]. The majority of bioethanol produced globally is made from biomass containing starch (from corn) or sucrose (from molasses or cane juice) [12]. However, bioethanol can also be made from non-edible lignocellulosic biomass like wood or agricultural residues [13].

Generally, the production of bioethanol from lignocellulosic biomass requires several steps: pretreatment, enzymatic hydrolysis to monomers (also called saccharification), fermentation, distillation, and dehydration [14]. The pretreatment phase can be performed using several different processes, such as concentrated acid hydrolysis, dilute acid hydrolysis, alkali treatment, sodium sulfite treatment, and steam explosion, among others [15]. The objective of the pretreatment is to remove lignin and improve the access of the enzymes to the cell wall carbohydrates (mainly cellulose) to obtain fermentable sugars [16]. The next step is saccharification, in which cellulases, typically from *Trichoderma* spp., are applied to produce fermentable monomer sugars from polysaccharides, primarily cellulose [14]. After saccharification, sugar monomers can be fermented to ethanol, typically with *Saccharomyces* spp., resulting in concentrations of 40–50 g/L [17].

Almost all commercially available lignocellulolytic enzymes needed for the hydrolysis step are produced by fermentation with the ascomycete *Trichoderma reesei* [18]. However, the purchase of this enzymes can make up 20–40% of the operating costs in a lignocellulosic biomass-based biorefinery. Accordingly, enzyme cost is a major determinant of the viability of commercial-scale production of bioethanol [19,20]. Onsite production of enzymes using low-value substrates could be an option to make a bioethanol facility more economical. In addition, using FMWs materials as a substrate for fungal cellulases production would make the biorefinery more environmentally sustainable.

In order to improve enzyme performance, extensive research has been carried out on the cellulolytic system of *T. reesei* to elucidate the enzymes and gene regulation involved in the utilization of lignocellulosic materials. Despite this, enzyme production from *T. reesei* still faces two major bottlenecks. The first one is because some of the produced enzymes have low catalytic activity under industrial conditions, due to the presence of high concentrations of inhibitory compounds generated from the degradation of carbohydrates and lignin during the pretreatment of lignocellulosic biomass [21,22]. The second one is because certain key enzymes are present in limited amounts [18,23]. Most research to date has focused on overcoming these bottlenecks by improving the performance of *T. reesei* secretion systems and investigating other *Trichoderma* spp. with higher secretion potential. Few studies have centered on evaluating enzyme production using less expensive lignocellulosic raw materials [24]. Consequently, the main objective of this research was to study the use of FMWs for cellulases production using an isolated fungal strain from olive stones. Production of cellulases from low-cost residues such as FMWS, could support future on-site production of cellulase enzymes from locally available waste materials for bioethanol production.

2. Materials and Methods

2.1. Substrate Preparation

FMWs were dried at 60 °C on a hot plate for at least 24 hours, avoiding burning. Subsequently, they were ground in a crushing machine and sieved through a 300 µm mesh (VWR, Radnor, PA, USA). This material was used at 0.5% as the carbon source for liquid and solid medium experiments.

2.2. Chemicals

Glycine, sodium hydroxide (NaOH), sodium deoxycholate (DOC), 3,5-dinitro-2-hydroxybenzoic acid (DNS), trichloroacetic acid (TCA), trifluoroacetic acid, ammonium carbonate, iodoacetamide, acetonitrile, potassium ferricyanide, sodium acetate, sodium thiosulfate, Congo red, carboxymethylcellulose, agar, hexadecyltrimethylammonium bromide (CTAB), glucose, acetic acid, NaCl, (NH₄)₂SO₄, KH₂PO₄, MgSO₄, FeSO₄ × 7H₂O, isopropanol, chloroform, Milli-Q water, isoamyl alcohol, acetone, and lactophenol (Merck, Darmstadt, Germany). CaCl₂, ZnSO₄, CoCl₂, and MnSO₄ (Phytotechnology Laboratories, Lenexa, KS, USA). Wizard®SV Gel and PCR Clean-Up System, PureYield™ Plasmid Miniprep System, pGEMT®-T Vector System I, and urea (Promega, Madison, WI, USA). Neopeptone, Luria Bertani (LB) broth, and potato dextrose agar (PDA) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). 4-nitrophenol-β-D-glucopyranoside (pNPG), 4-nitrophenol, Folin–Ciocalteu reagent, dithiothreitol, trypsin, and RNase (Sigma-Aldrich, Missouri, WI, USA). Agarose (Fermelo Biotec, Santiago, Chile).

2.3. Fungal Strain and Culture Conditions

The fungal strain was isolated from olive stones kept at room temperature. Later, it was laid onto PDA plates. The plates were incubated at 30 °C for 1 week, and plugs of PDA were extracted from the edge of the mycelium and placed on a new PDA plate. This procedure was repeated several times to obtain homogeneous colony formation. For the mycelium staining procedure, the fungal slide procedure was used. Briefly, a plug of a 0.4 mm² PDA was deposited on a glass slide. The mycelium was deposited on the plug and covered with a microscope coverslip. After growth of the mycelium on the PDA plug and the surface of the coverslip, this was carefully removed, and the mycelium was stained with a solution of lactophenol cotton blue and observed using an optical microscope (Optika, Lombardy, Italy). Liquid cultures were performed in Mandels medium as described previously [25]. In this step, a plate containing the mycelium was scraped using an inoculation loop. The mycelium was dissolved in an Erlenmeyer flask containing a sterile 0.9% NaCl solution and glass beads to facilitate spore release. After shaking, this solution was filtered using sterile dressing into an empty sterile flask. Spores were counted in a Petroff–Hauser chamber, and 4 × 10⁶ spores were inoculated in 100 mL of Mandel medium supplemented with 0.5% FMWs as the only carbon source. For solid-medium experiments, a 0.4 mm² area of a PDA plug containing the mycelium was extracted and placed on the surface of plates containing Mandel medium, 4% agar, and 0.5% FMWs. Mycelium radial growth was monitored each day by measurement of the colony diameter [26]. For the analysis of the presence of fungal cellulases on the plates, the Congo red protocol was performed according to Zeng et al. [27].

2.4. Strain Identification

The mycelium was grown in LB media at 30 °C for 5 days. Then, 1 mL of culture was centrifuged, resuspended in 800 µL of CTAB solution, and subsequently lysed in Precellys®24 (Bertin, Provenza, France) using 0.5 mm beads. After lysis, the tubes were kept at 65 °C for 30 min, and a mixture of chloroform/isoamyl alcohol was added. The samples were centrifuged at 13,000 rpm for 11 min at 25 °C. The supernatants were transferred to clean tubes, and an equal volume of cold isopropanol was added. The tubes were kept at –20 °C for 2 h, then centrifuged at 13,000 rpm for 11 min at 4 °C. The supernatants were discarded, and the pellets were air-dried at room temperature and then resuspended in Milli-Q water. RNase (10 mg/mL) was added to the tubes, which were incubated at 37 °C for 30 min. One volume

of chloroform/isoamyl alcohol was added, and the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants were extracted, and 2.5 volumes of cold ethanol were added to the tubes, which were kept at −20 °C for 30 min. The samples were centrifuged at 13,000 rpm for 10 min, and the pellets were air-dried at room temperature. DNA was resuspended in Milli-Q water, quantified using Nanodrop 2000 (Thermo Scientific, Delaware, USA), and visualized using 1% agarose gel electrophoresis. Aliquots were used in PCR reactions using ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers [26]. The obtained PCR fragment corresponding to the ITS1-5.8-ITS2 ribosomal region was purified using Wizard®SV Gel and PCR Clean-Up System, cloned in pGEMT®-T Vector System I, and used to transform JM101 competent cells. Positive colonies containing the fragment were verified with colony PCR using T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') primers. A white colony was inoculated in LB medium for plasmid DNA purification using PureYield™ Plasmid Miniprep System. An aliquot of this sample was sequenced using T7 and SP6 primers (Macrogen, Seoul, Korea). The sequence was edited with Editseq software (DNASTAR, Madison, WI, USA) and compared with sequences from GenBank using BLAST algorithm (Megablast) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences were aligned with ClustalW and analyzed with the MEGA-7 software [28]. Phylogenetic analyses were carried out using the neighbor-joining method with the Kimura-2-parameter model [29]. There were 569 positions in the final dataset. Bootstrap analyses were performed on 10,000 replicates.

2.5. Enzyme Activity

Filter paper assays were done using 50 mg of Whatman grade 1 paper introduced in a glass tube in the presence of 1 mL of 50 mM citrate buffer pH 5.0 and 0.5 mL of enzyme. The sample was incubated at 50 °C for 1 h, and reducing sugars were quantified by the DNS method. Glucose in the range 0.2 µg/µL to 2 µg/µL was used as a standard. The samples were measured at 540 nm [30]. β-glucosidase activity was determined using 1.5 mg/mL of 4-nitrophenol-β-D-glucopyranoside (pNPG) in the presence of 20 mM sodium acetate buffer pH 3.5. Then, 250 µL of enzyme was added to the mixture, and the samples were incubated at 50 °C for 10 min. Then the reaction was stopped by addition of 500 µL of 0.4 M glycine/NaOH buffer, pH 10.7, and the mixture was measured at 405 nm. The standard curve was measured using 4-nitrophenol in the range of 15 to 75 µM. All activities were expressed in international units (U), corresponding to the quantity of enzyme hydrolyzing 1 µmol of the substrate in 1 min. Triplicate assays were performed. Total protein content was determined using the Lowry method modified by Tan [31], using bovine serum albumin in the range of 0.02 µg/µL to 0.2 µg/µL as a standard and measuring samples at 660 nm. Specific enzyme activity in both filter paper and β-glucosidase activity assays is expressed as U/mg total protein.

2.6. Sample Preparation and SDS-PAGE

For the preparation of the extracellular protein fractions, cultures from the fifth day were harvested by centrifugation at 4000 rpm for 30 min at 4 °C, and the supernatants were filtered through a 0.22 µm filter (Millipore, Burlington, MA, USA). A total of 10 µg of protein was precipitated by the DOC-TCA method and collected by centrifugation (13,000 rpm, 5 min, 4 °C). The resulting pellet was washed two times with cold acetone and air-dried. The protein pellet was resuspended in loading buffer, boiled, and loaded onto 12% SDS-PAGE (denaturant electrophoresis). The gel was run at a constant voltage of 200 V for 2 h. After running, the gel was stained with silver according to Schevchenko et al. [32]. Gels from two different biological replicates were used for sequencing by means of mass spectrometry.

2.7. Peptide Preparation for Mass Spectrometry

For each lane of gel, 10 zones were selected for cutting, and each excised band was destained with a 1:1 mixture of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. After washing with Milli-Q water, a mixture of 1:1 ammonium carbonate and acetonitrile was added for 2 h. Then, acetonitrile was added until drying of the gel piece, which was hydrated in 0.1 M ammonium

carbonate for 5 min at room temperature. An equal volume of acetonitrile was added, and the sample was incubated for 15 min at 37 °C; then, the liquid was removed, and the tube was dried in a speed vac. Gel pieces were reduced with 10 mM dithiothreitol in 0.1 M ammonium carbonate at 56 °C for 45 min. The liquid was discarded, and the gel was incubated in 0.1 M ammonium carbonate and 55 mM iodoacetamide for 30 min. The gel was washed two times with a 1:1 mixture of ammonium carbonate and acetonitrile for 15 min and dried in a speed vac. The dried samples were incubated with 10 ng of trypsin in ammonium carbonate solution overnight. After digestion, the samples were incubated with a mixture of 25 mM ammonium carbonate and acetonitrile for 50 min in a sonicator bath. Then, the samples were incubated in mixtures composed of 30% acetonitrile/0.1% trifluoroacetic acid and 50% acetonitrile/0.1% trifluoroacetic acid. Each supernatant was collected and concentrated in the speed vac for 30 min. Finally, 20 µL of trifluoroacetic acid was added, and the solutions were purified by Ziptip (Millipore, Burlington, MA, USA).

2.8. Mass Spectrometry

Dried samples were dissolved in 10 µL of 2% acetonitrile/0.1% trifluoroacetic acid and applied to an Ultimate 3000 Nano-HPLC (Dionex, Idstein, Germany). Each sample was first trapped on a 1 mm PepMap-trapping column (Dionex, Idstein, Germany) for 10 min at 30 µL/min of 2% acetonitrile/0.1% trifluoroacetic acid and subsequently subjected to a 75 µm ID, 5 cm PepMap C18-column (Dionex, Idstein, Germany). Peptide separation was performed by an acetonitrile gradient at 300 nL/min. The separation column outlet was coupled online to a nano-spray interface of an Esquire HCT ETDII-ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectra were acquired in positive MS-mode, tuned for tryptic peptides. MS/MS-precursor selection was performed in an optimized automatic regime, with preference for double- and triple-charged ions. Every selected precursor was fragmented by collision-induced dissociation (CID) and electron transfer dissociation (ETD), respectively. MS/MS spectra were processed by the Data Analysis and BioTools software from Bruker. Combined CID/ETD-derived fragment lists were analyzed by the MASCOT algorithm in in-house and Swissprot databases, generating a presence-absence table. The total number of proteins belonging to the fungi kingdom were counted for each treatment. Cellulases were selected to construct a final table.

3. Results and Discussion

A fungal strain was isolated from olive stones kept at room temperature. After successive passages onto PDA plates, a homogenous fluffy-type mycelium of white color was observed which turned green after 5 days of cultivation (Figure 1a). Examination of samples of the lactophenol-cotton-blue-stained mycelium under optical microscopy showed the presence of segmented hyphae, tree-like reproductive structure, and conidia showing round to ovoidal form. Similar features are found in *Trichoderma* species (Figure 1b,c) [33].

PCR amplification using ITS-1 and ITS-4 primers and the mycelium DNA produced a fragment of around 570 bp (Figure 2a). This fragment was cloned in pGEM-T vector and used for transformation of *Escherichia coli* competent cells. After selection of colonies containing the recombinant plasmid, positive colonies were inoculated in LB medium for plasmid purification. Plasmids containing the PCR product were further sequenced using the T7 and SP6 vector primers. The amplified ITS1-5.8S-ITS2 sequence from strain FG displayed 100% homology to the same region of several strains of *Trichoderma asperellum* associated with plants worldwide. Also, this region is 99% identical to that of *T. asperellum* UC 1 (Genbank ID: MF774876) isolated in Malaysia, which is reported to be a high cellulase and xylanase producer [34]. A phylogenetic tree was constructed using these data and related sequences extracted from the NCBI database, as shown in Figure 2b. This figure shows that strain FG sequence was located in a clade together with other *Trichoderma* species. These data suggest that strain FG belongs to *Trichoderma* spp.

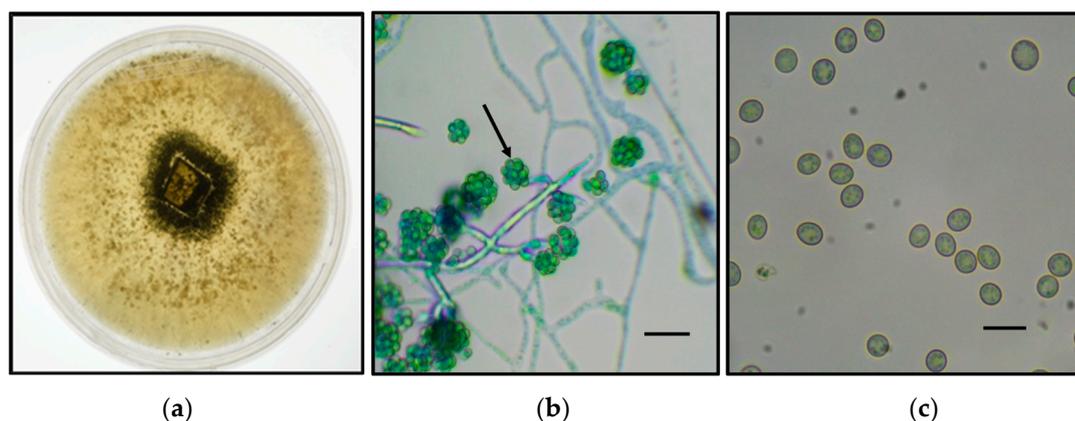


Figure 1. Morphology of strain FG. (a) Mycelium from strain FG grown on a potato dextrose agar (PDA) plate for 5 d; (b) A sample taken from a mycelium grown on a PDA plate and stained with lactophenol cotton blue. This sample was observed under an optical microscope using 40× magnification. Bar indicates 20 μm. Tree-like structures containing conidia can be observed (black arrow); (c) Microscopic observation of spores by an optical microscope using 100× magnification. Bar indicates 5 μm.

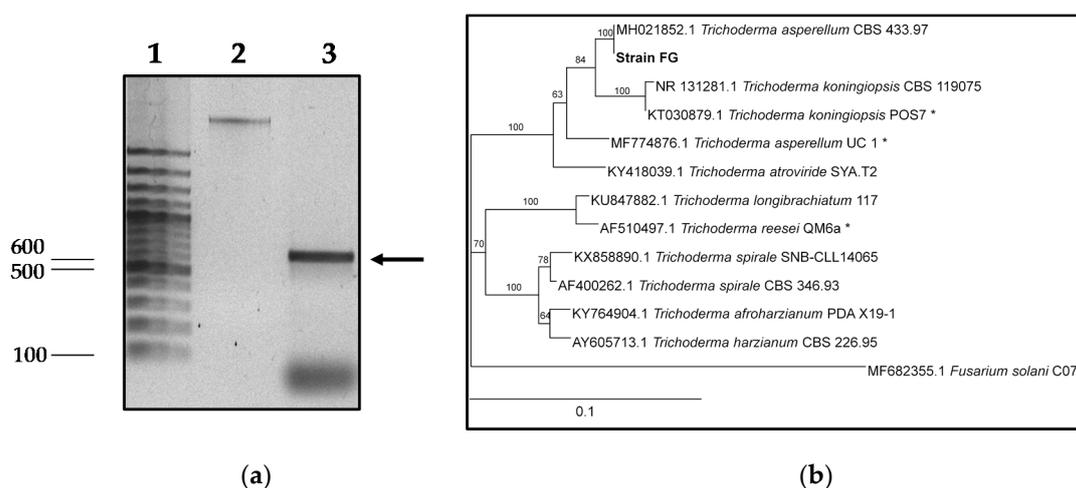


Figure 2. Molecular analysis of strain FG. (a) Gel electrophoresis of strain FG DNA. Lane 1: Molecular marker 100 bp; lane 2: genomic DNA from strain FG extracted with the hexadecyltrimethylammonium bromide (CTAB) method; lane 3: PCR product using ITS primers and genomic DNA as a template. Arrow indicates the PCR product of the expected size (~570 bp); (b) Neighbor-joining analysis with the Kimura-2-parameter model after aligning ITS1-5.8-ITS2 sequences with Clustal X in MEGA7. Bootstrap analyses were performed for 10,000 replicates. The ITS1-5.8-ITS2 sequence from *Fusarium solani* was used as outgroup. Asterisk indicates fungal strains characterized as high cellulases producers [18,34–38].

To evaluate the behavior of the *Trichoderma* sp. on FMWs, the fungus was grown on both solid and liquid Mandel medium supplemented with 0.5% FMWs. For comparison purposes, glucose was added to a fourth set of liquid cultures and plates. On these plates, *Trichoderma* sp. growth was observed on the three FMWs, however the highest radius value at 72 h was observed in OMSW and GP plates (4.3 cm) followed by glucose (4.1 cm) and TP (4.0 cm) (Figure 3) plates.

The samples were incubated in 100 mL liquid cultures at 30 °C, and the total biomass (wet weight) obtained after 5 d of culture was 17.4 g for glucose, 11.0 g for OMSW, 9.8 g for G,P and 8.4 g for TP. In addition, the final protein concentrations in these samples were 0.6, 0.7, 0.3, and 0.4 mg/mL, respectively. The visual inspection of the FMW cultures on the fifth day revealed incomplete degradation; however, OMSW seemed to be the most preferable carbon source among the three FMWs.

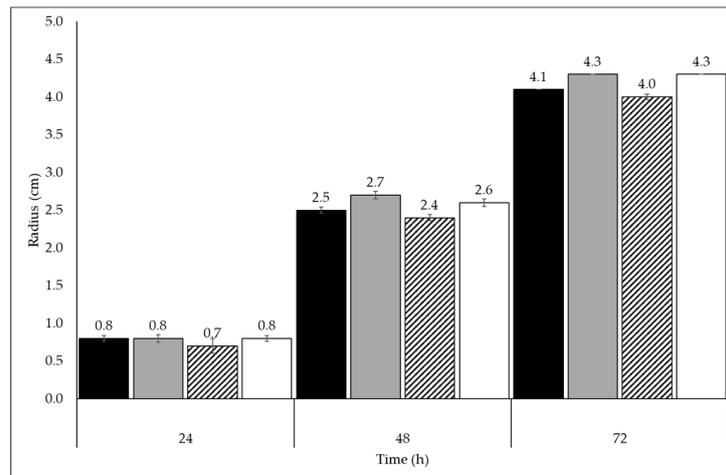


Figure 3. Radial growth of the *Trichoderma* sp. on FMWs plates. *Trichoderma* sp. was grown on Mandels medium supplemented with glucose (black bar), olive-mill solid waste (OMSW, gray bar), tomato pomace (TP, striped bar), and grape pomace (GP, white bar) at 0.5% as the sole carbon source. Error bars indicate the standard deviations (SDs) based on three different replicated experimental values. Numbers on the top of each bar indicate the average radius corresponding to mycelial growth.

Fungal growth was evidenced when the fungal strain was inoculated on plates containing LB medium with 1% carboxymethyl cellulose stained with Congo red solution. Initially, the plates were fully stained red. Once the cellulose was degraded after 5 d of incubation, the red color disappeared leaving a pale-colored halo, which was indicative of the presence of cellulases released into the culture medium (Figure 4a). This preliminary evidence prompted us to search for cellulases when the fungi were grown in FMWs.

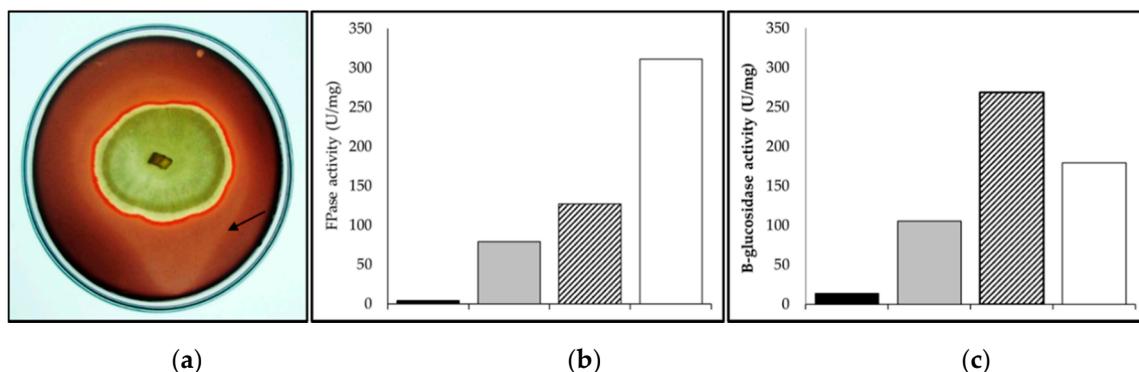


Figure 4. Cellulolytic activities of culture supernatants from the *Trichoderma* sp. grown on different food manufacturing wastes (FMWs). (a) The *Trichoderma* sp. was grown on a Petri plate containing carboxymethyl cellulose and stained with Congo red solution. The white halo is indicative of the presence of cellulases released into the culture medium. The arrow indicates the lack of staining due to the activity of cellulases secreted by the fungus (b) Filter paper activity (FPase) from culture supernatants (c) β -glucosidase activity from culture supernatants. For b and c, the culture supernatants were extracted on the fifth day of growth in Mandel medium supplemented with glucose (black bar), OMSW (gray bar), TP (striped bar), and GP (white bar). Activities are expressed in mg of total protein. For FPAase, the activity, expressed in U/mL, was 2.6, 63.2, 33.0, and 112.0, respectively. For β -glucosidase, it was 0.2, 1.7, 1.6, and 1.4 U/mL, respectively.

Culture supernatants prepared from FMWs liquid cultures after 5 d of fungal growth were evaluated for their total cellulase activity (i.e., endo- β -1,4-D-glucanase, 1,4- β -D-glucan

cellobiohydrolase, and β -glucosidase) and β -glucosidase activities through filter paper and pNPG assays, respectively. Filter paper specific activity was clearly higher in the FMW culture supernatants in comparison to the glucose supernatant, with the GP culture supernatant having the highest value (Figure 4b). The same trend was observed for β -glucosidase specific activities, although the TP supernatant had the highest value (Figure 4c).

To confirm the presence of cellulases, proteins were extracted from the culture supernatants of the *Trichoderma* sp. grown on glucose and FMWs on the fifth day. Proteins were separated by SDS-PAGE (denaturant electrophoresis) and analyzed by mass spectrometry. This qualitative analysis identified 336, 219, 244, and 210 proteins in the supernatant media from cells grown in glucose, OMSW, GP, and TP, respectively (results not shown). The identity of the proteins in each fraction is found in Table 1, and a detailed description is given in Supplementary Material (ST1–ST8). In total, 24 distinct cellulases were found in 4 four different residues. Endo- β -1,4-D-glucanase, 1,4- β -D-glucan cellobiohydrolase and β -glucosidases were found in the culture supernatants of FMWs, whereas the glucose supernatants did not contain any exo- or endo-glucanases.

Table 1. Cellulases found in the culture supernatants of the *Trichoderma* sp. grown on Mandel medium supplemented with glucose or FMWs.

E.C. Number	GH Family	Predicted Protein	Glucose	OMSW	TP	GP
Endoglucanases						
3.2.1.4	GH7	Endo- β -1,4-D-glucanase EG-1		x		x
Exoglucanases						
3.2.1.91	GH7	Probable 1,4- β -D-glucan cellobiohydrolase A		x		x
3.2.1.91	GH7	Probable 1,4- β -D-glucan cellobiohydrolase B		x		x
3.2.1.91		Probable 1,4- β -D-glucan cellobiohydrolase C		x	x	x
3.2.1.91		1,4- β -D-glucan cellobiohydrolase C		x	x	
3.2.1.91		1,4- β -D-glucan cellobiohydrolase A				x
3.2.1.91		1,4- β -D-glucan cellobiohydrolase CEL6A				x
3.2.1.91		1,4- β -D-glucan cellobiohydrolase B		x		x
3.2.1.91		1,4- β -D-glucan cellobiohydrolase 1		x	x	x
3.2.1.91	GH7	Putative 1,4- β -D-glucan cellobiohydrolase type C		x		x
3.2.1.91	GH6	1,4- β -D-glucan cellobiohydrolase 3		x	x	
β-glucosidases						
3.2.1.21	GH3	Probable β -glucosidase E		x		
3.2.1.21		Probable β -glucosidase M	x			x
3.2.1.21		Probable β -glucosidase G	x	x		x
3.2.1.21		Probable β -glucosidase L		x	x	x
3.2.1.21		Probable β -glucosidase F		x	x	x
3.2.1.21		Probable β -glucosidase A	x	x	x	x
3.2.1.21	GH3	Probable β -glucosidase ARB_05654	x	x		x
3.2.1.21	GH3	β -glucosidase cel3A		x	x	x
3.2.1.21	GH3	β -glucosidase 1	x			

The ability of species belonging to genus *Trichoderma* to grow in complex substrates has been previously reported. Using a synthetic medium, containing cellulose as the sole carbon source, Do Vale et al. identified few cellulases in the culture supernatant of *Trichoderma harzianum* [36]. In contrast, after growth of *T. harzianum* using the same medium supplemented with sugarcane bagasse, Gomez-Mendoza et al. found that this complex substrate induced a higher secretion of cellulolytic and xylanolytic activities when compared with other substrates such as glucose [37]. In a comparative study of the extracellular proteins produced by *T. asperellum* S4F8 and *T. reesei* Rut C30 after solid-state fermentation on the same substrate, Marx et al. found 14 and 12 cellulases, respectively [38]. In a study using *Aspergillus niger* and *T. reesei* grown on sugarcane with two levels of cell wall complexity, culm in natura and pretreated, Borin et al. found that although *A. niger* and *T. reesei* both produced cellulases, *A. niger* produced two times more enzymes than *T. reesei* [39].

In general, the activities from the culture supernatants agreed with the mass spectrometry data. Glucose was found repressive as expected; however, five β -glucosidases were still found in the culture

supernatant of this simple substrate. These results are similar to those of Gómez-Mendoza et al. who found that *T. harzianum* cultured with glucose presented two β -glucosidases [37]. These enzymes might be responsible for the residual activities seen in Figure 4c.

Only one endo- β -1,4-D-glucanase was found in our analysis. However, 14 1,4- β -D-glucan cellobiohydrolases were found in the culture supernatants from FMWs samples. The cellulolytic system of *T. reesei* has been studied in detail. It is composed by seven endo- β -1,4-D-glucanases, two 1,4- β -D-glucan cellobiohydrolases, and seven β -D-glucosidases. The endo- β -1,4-D-glucanases belong to the GH5, GH7, GH12, and GH45 families, and the 1,4- β -D-glucan cellobiohydrolases belong to the GH6 and GH7 families. Finally, the β -glucosidases belongs to the GH3 family [18,23,40]. In the cellulose degradation model by enzymes from *T. reesei*, the enzyme endo- β -1,4-D-glucanases acts by cleaving off the amorphous regions of the chain, while the 1,4- β -D-glucan cellobiohydrolases cleave off at the nonreducing and reducing ends. The oligosaccharide products from this hydrolysis are further hydrolyzed into monosaccharides by β -glucosidase into glucose. Other accessory enzymes such as lytic polysaccharide monooxygenases and swollenin, which expands cellulose chains to improve enzyme accessibility, were not found in our four culture conditions [18,23,40].

From a biorefinery prospective, enzyme cost is one of the main drivers of the viability of commercial-scale production of bioethanol, representing around 20–40% of the operating costs in a lignocellulosic biomass-based biorefinery [19,22]. Using low-value substrates to produce enzymes onsite could be an option to make a bio-ethanol facility more sustainable and economical. Currently, most economic studies assume purchasing cellulases from an enzyme manufacturer [19,41,42]. Although this approach avoids the capital cost of on-site enzyme production, it includes the transportation of the enzyme to the biorefinery facility, adding a non-trivial amount to the enzyme cost [43]. Humbird et al. modeled on-site enzyme generation for ethanol production and found that the carbon source makes up 57% of the cost of enzyme production, followed by 21% capital cost, 13% of electricity, and 9% of other nutrients and fixed costs [44]. Consequently, the utilization of less expensive carbon sources like agroindustrial residues such as OMSW, GP, and TP will allow reducing the cost of enzymes, thereby reducing ethanol production costs.

This investigation suggests that *Trichoderma* sp. can grow on a wide range of carbon sources, including complex substrates such as FMWs. This enables a great variability in the production of cellulases and other enzymes (data not shown), since gene expression and enzyme secretion are directly dependent on the substrate being consumed. Accordingly, in FMWs, whose full chemical composition is still unknown, sugars released during fungal hydrolysis might induce or repress cellulase genes at different levels. Some cellulase inducers are cellulose, β -glucan, xylan, lactose, cellobiose, and sophorose; glucose is the main cellulase repressor [40].

4. Conclusions

An isolated fungal strain found on olive stones was classified as a *Trichoderma* sp. and exhibited cellulolytic activity with no addition of glucose on synthetic solid media and in liquid media. Additionally, the cellulolytic activity increased when the *Trichoderma* sp. was grown on FMWs. The presence of all three types of cellulases in the FMWs culture supernatants was verified by mass spectrometry analysis. The ability of the *Trichoderma* sp. to grow and produce cellulolytic enzymes in the presence of FMWs could enable the onsite production of cellulase enzymes in lignocellulosic bioethanol biorefineries. This would improve biorefinery economics by avoiding the purchase of commercial enzymes, while improving their environmental sustainability by utilizing waste materials.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/9/20/4419/s1>, Table S1: Glucose. Replicate 1; Table S2: Glucose. Replicate 2; Table S3: OMSW. Replicate 1; Table S4: OMSW. Replicate 2; Table S5: TP. Replicate 1; Table S6: TP. Replicate 2; Table S7: GP. Replicate 1; Table S8: GP. Replicate 2.

Author Contributions: Conceptualization, F.G.-F.; Investigation, F.G.-F., S.C.-O., C.F. and T.K.; Methodology, F.G.-F., A.E.-V. and R.M.-V.; Writing—original draft, F.G.-F.; Writing—review & editing, F.G.-F., A.E.-V. and R.M.-V.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Khan, F.I.; Husain, T.; Hejazi, R. An overview and analysis of site remediation technologies. *J. Environ. Manag.* **2004**, *71*, 95–122. [[CrossRef](#)]
2. Gill, R.T.; Harbottle, M.J.; Smith, J.W.N.; Thornton, S.F. Electrokinetic-enhanced bioremediation of organic contaminants: A review of processes and environmental applications. *Chemosphere* **2014**, *107*, 31–42. [[CrossRef](#)]
3. Alburquerque, J. Agrochemical characterisation of “alperujo”, a solid by-product of the two-phase centrifugation method for olive oil extraction. *Bioresour. Technol.* **2004**, *91*, 195–200. [[CrossRef](#)]
4. Rubio-Senent, F.; Lama-Muñoz, A.; Rodríguez-Gutiérrez, G.; Fernández-Bolaños, J. Isolation and identification of phenolic glucosides from thermally treated olive oil byproducts. *J. Agric. Food Chem.* **2013**, *61*, 1235–1248. [[CrossRef](#)]
5. Del Valle, M.; Cámara, M.; Torija, M.E. Chemical characterization of tomato pomace. *J. Sci. Food Agric.* **2006**, *86*, 1232–1236. [[CrossRef](#)]
6. Deng, Q.; Penner, M.H.; Zhao, Y. Chemical composition of dietary fiber and polyphenols of five different varieties of wine grape pomace skins. *Food Res. Int.* **2011**, *44*, 2712–2720. [[CrossRef](#)]
7. Saini, J.K.; Saini, R.; Tewari, L. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech* **2015**, *5*, 337–353. [[CrossRef](#)]
8. Sánchez, C. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnol. Adv.* **2009**, *27*, 185–194. [[CrossRef](#)]
9. Manavalan, T.; Manavalan, A.; Heese, K. Characterization of Lignocellulolytic Enzymes from White-Rot Fungi. *Curr. Microbiol.* **2015**, *70*, 485–498. [[CrossRef](#)]
10. Jayasekara, S.; Ratnayake, R. Microbial Cellulases: An Overview and Applications. In *Cellulose*; IntechOpen: London, UK, 2019; p. 84531.
11. Kuhad, R.C.; Gupta, R.; Singh, A. Microbial cellulases and their industrial applications. *Enzyme Res.* **2011**, *2011*, 280696. [[CrossRef](#)]
12. Bušić, A.; Mardetko, N.; Kundas, S.; Morzak, G.; Belskaya, H.; Ivancic Santek, M.; Komes, S.; Novak, S.; Santek, B. Bioethanol Production from Renewable Raw Materials and Its Separation and Purification: A Review. *Food Technol. Biotechnol.* **2018**, *56*, 289–311.
13. Duwe, A.; Tippkötter, N.; Ulber, R. Lignocellulose-Biorefinery: Ethanol-Focused. In *Advances in Biochemical Engineering/Biotechnology*; Scheper, T., Ed.; Springer: Berlin, Germany, 2017; Volume 166, pp. 177–215.
14. Robak, K.; Balcerak, M. Review of Second Generation Bioethanol Production from Residual Biomass. *Food Technol. Biotechnol.* **2018**, *56*, 174–187. [[CrossRef](#)]
15. Jönsson, L.J.; Martín, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresour. Technol.* **2016**, *199*, 103–112. [[CrossRef](#)]
16. Ewanick, S.; Bura, R. *Hydrothermal Pretreatment of Lignocellulosic Biomass*; Woodhead Publications Ltd.: Cambridge, UK, 2010.
17. Kumar, A.K.; Sharma, S. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review. *Bioresour. Bioprocess.* **2017**, *4*, 7. [[CrossRef](#)]
18. Bischof, R.H.; Ramoni, J.; Seiboth, B. Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. *Microb. Cell Fact.* **2016**, *15*, 106. [[CrossRef](#)]
19. Juneja, A.; Kumar, D.; Murthy, G. Economic feasibility and environmental life cycle assessment of ethanol production from lignocellulosic feedstock in Pacific Northwest US. *J. Renew. Sustain. Energy* **2013**, *5*, 023142. [[CrossRef](#)]
20. Barta, Z.; Sassner, P.; Zacchi, G.; Reczey, K. Technoeconomic aspects of on-site cellulase production. *Hung. J. Ind. Chem.* **2008**, *36*, 5–9.
21. Cantarella, M.; Cantarella, L.; Gallifuoco, A.; Spera, A.; Alfani, F. Effect of Inhibitors Released during Steam-Explosion Treatment of Poplar Wood on Subsequent Enzymatic Hydrolysis and SSF. *Biotechnol. Progress* **2004**, *20*, 200–206. [[CrossRef](#)]

22. García-Aparicio, M.P.; Ballesteros, I.; González, A.; Oliva, J.M.; Ballesteros, M.; Negro, M.J. Effect of Inhibitors Released During Steam-Explosion Pretreatment of Barley Straw on Enzymatic Hydrolysis. *Appl. Biochem. Biotechnol.* **2006**, *129*, 278–288. [[CrossRef](#)]
23. Druzhinina, I.S.; Kubicek, C.P. Genetic engineering of *Trichoderma reesei* cellulases and their production. *Microb. Biotechnol.* **2017**, *10*, 1485–1499. [[CrossRef](#)]
24. Merino, S.T.; Cherry, J. Progress and challenges in enzyme development for biomass utilization. *Adv. Biochem. Eng. Biotechnol.* **2007**, 95–120.
25. Ryu, D.D.Y.; Mandels, M. Cellulases: Biosynthesis and applications. *Enzyme Microb. Technol.* **1980**, *2*, 91–102. [[CrossRef](#)]
26. Gordillo, F.; Candia, O.; Gonzalez, A.R.; Pradel, P.; Cordero, C.; Diaz, R.; Garcia-Gonzalez, R. Degradation of Food Manufacturing Wastes by a Fungal Isolate. *Chil. J. Agric. Anim. Sci.* **2017**, *33*, 84–90. [[CrossRef](#)]
27. Zeng, R.; Yin, X.-Y.; Ruan, T.; Hu, Q.; Hou, Y.-L.; Zuo, Z.-Y.; Huang, H.; Yang, Z.-H. A Novel Cellulase Produced by a Newly Isolated *Trichoderma virens*. *Bioengineering* **2016**, *3*, 13. [[CrossRef](#)]
28. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [[CrossRef](#)]
29. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [[CrossRef](#)]
30. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. [[CrossRef](#)]
31. Tan, L.U.L.; Chan, M.K.-H.; Saddler, J.N. A modification of the Lowry method for detecting protein in media containing lignocellulosic substrates. *Biotechnol. Lett.* **1984**, *6*, 199–204. [[CrossRef](#)]
32. Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850–858. [[CrossRef](#)]
33. Torres-De la Cruz, M.; Ortiz-García, C.F.; Bautista-Muñoz, C.; Ramírez-Pool, J.A.; Ávalos-Contreras, N.; Cappello-García, S.; De la Cruz-Pérez, A. Diversidad de *Trichoderma* en el agroecosistema cacao del estado de Tabasco, México. *Rev. Mex. Biodivers.* **2015**, *86*, 947–961. [[CrossRef](#)]
34. Ezeilo, U.R.; Lee, C.T.; Huyop, F.; Zakaria, I.I.; Wahab, R.A. Raw oil palm frond leaves as cost-effective substrate for cellulase and xylanase productions by *Trichoderma asperellum* UC1 under solid-state fermentation. *J. Environ. Manag.* **2019**, *243*, 206–217. [[CrossRef](#)]
35. Castrillo, M.L.; Bich, G.Á.; Modenutti, C.; Turjanski, A.; Zapata, P.D.; Villalba, L.L. First whole-genome shotgun sequence of a promising cellulase secretor, *Trichoderma koningiopsis* Strain POS7. *Genome Announc.* **2017**, *5*, e00823–e00917. [[CrossRef](#)]
36. Do Vale, L.H.F.; Gómez-Mendoza, D.P.; Kim, M.S.; Pandey, A.; Ricart, C.A.O.; Edivaldo, X.F.F.; Sousa, M.V. Secretome analysis of the fungus *Trichoderma harzianum* grown on cellulose. *Proteomics* **2012**, *12*, 2716–2728. [[CrossRef](#)]
37. Gómez-Mendoza, D.P.; Junqueira, M.; Do Vale, L.H.F.; Domont, G.B.; Ferreira Filho, E.X.; de Sousa, M.V.; Ricart, C.A.O. Secretomic survey of *Trichoderma harzianum* grown on plant biomass substrates. *J. Proteome Res.* **2014**, *13*, 1810–1822. [[CrossRef](#)]
38. Marx, I.J.; Van Wyk, N.; Smit, S.; Jacobson, D.; Viljoen-Bloom, M.; Volschenk, H. Comparative secretome analysis of *Trichoderma asperellum* S4F8 and *Trichoderma reesei* Rut C30 during solid-state fermentation on sugarcane bagasse. *Biotechnol. Biofuels* **2013**, *6*, 172. [[CrossRef](#)]
39. Borin, G.P.; Sanchez, C.C.; de Souza, A.P.; de Santana, E.S.; de Souza, A.T.; Leme, A.F.P.; Squina, F.M.; Buckeridge, M.; Goldman, G.H.; de Castro Oliveira, J.V. Comparative Secretome Analysis of *Trichoderma reesei* and *Aspergillus niger* during Growth on Sugarcane Biomass. *PLoS ONE* **2015**, *10*, e0129275. [[CrossRef](#)]
40. De Paula, R.G.; Antoniêto, A.C.C.; Ribeiro, L.F.C.; Carraro, C.B.; Nogueira, K.M.V.; Lopes, D.C.B.; Costa Silva, A.; Zerbini, M.T.; Pedersoli, W.R.; Do Nascimento Costa, M.; et al. New genomic approaches to enhance biomass degradation by the industrial fungus *Trichoderma reesei*. *Int. J. Genomics* **2018**, *2018*, 1974151. [[CrossRef](#)]
41. Joelsson, E.; Borbála, E.; Mats, G.; Ola, W. Biotechnology for Biofuels Techno-Economic Evaluation of Integrated First- and Second-Generation Ethanol Production from Grain and Straw. *Biotechnol. Biofuels* **2016**, *9*, 1. [[CrossRef](#)]
42. Littlewood, J.; Wang, L.; Turnbull, C.; Murphy, R.J. Techno-economic potential of bioethanol from bamboo in China. *Biotechnol. Biofuels* **2013**, *6*, 173. [[CrossRef](#)]

43. Davis, R.; Grundl, N.; Tao, L.; Bidy, M.J.; Tan, E.C.D.; Beckham, G.T.; Humbird, D.; Thompson, D.N.; Roni, M.S. *Process Design and Economics for the Conversion of Lignocellulosic Biomass to Hydrocarbon Fuels and Coproducts: Biochemical Design Case Update: Biochemical Deconstruction and Conversion of Biomass to Fuels and Products via Integrated Biorefinery Pathways*; NREL/TP-5100-71949; National Renewable Energy Laboratory: Golden, CO, USA, 2018.
44. Humbird, D.; Davis, R.; Tao, L.; Kinchin, C.; Hsu, D.; Aden, A.; Schoen, P.; Lukas, J.; Olthof, B.; Worley, M.; et al. *Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol*; NREL/TP-5100-47764; National Renewable Energy Laboratory: Golden, CO, USA, 2011.



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).