applied sciences

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Lignocellulolytic Potential of the Recently Described Species *Aspergillus olivimuriae* on Different Solid Wastes

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Abstract: The genus *Aspergillus* encompasses several species with relevant lignocellulose-degrading capacity, and a novel species, denominated *A. olivimuriae*, was recently discovered after its isolation from table olive brine. The acquisition of insight into this species and the assessment of its potential relied on a bioinformatics approach, based on the CAZy database, associated with enzymatic activity profiles in solid-state cultures on four different types of waste, including residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction and barley straw. The CAZy analysis of *A. olivimuriae* genome showed that the number of predicted genes for each family was close to that of other *Aspergillus* species, except for cellobiose dehydrogenase, acetyl xylan esterase and polygalacturonases. In *A. olivimuriae* solid-state cultures, hemicellulose degradation outperformed that of cellulose, and lignin removal did not occur, regardless of the growth substrate. This is in line with its CAZy content and the extent of hemicellulolytic, and ligninolytic activities detected in its solid-state cultures. RTB and barley straw were the substrates enabling the best glycosyl hydrolase production levels. The exception was SCG, the hemicellulose composition of which, mainly made of glucomannans and galactomanans, led to the highest β-mannanase and β-mannosidase production levels (3.72 ± 0.20 and 0.90 ± 0.04 IU g^{-1} substrate, respectively).

Keywords: *Aspergillus olivimuriae*; solid-state fermentation; wastes; supply chains; glycosyl hydrolases; CAZyme annotation

Citation: Carota, E.; Crognale, S.; Russo, C.; Petruccioli, M.; D'Annibale, A. Lignocellulolytic Potential of the Recently Described Species *Aspergillus olivimuriae* on Different Solid Wastes. *Appl. Sci.* **2021**, *11*, 5349. https://doi.org/10.3390/app11125349

Academic Editor: Antonio Valero

Received: 6 May 2021
Accepted: 7 June 2021
Published: 9 June 2021

1. Introduction

The characterization of new isolates and strains from public culture collections is essential for knowledge implementation and to provide the research and biotechnological industry with helpful information for conducting screenings to find microbial strains of potential interest. There is a global abundance of lignocellulosic residues, for instance, lignocellulolytic fungi are acquiring an ever-increasing role in biorefinery-based applications aiming to upgrade biomass through the production of value-added products [1]. Owing to the structural heterogeneity and complexity that characterize plant biomass, fungi need to produce a wide set of glycosyl hydrolase activities to perform the breakdown of plant polysaccharides into their monomeric constituents [2,3]. The Carbohydrate-Active EnZyme database (CAZy; http://www.cazy.org; accessed on 8 June 2021) has divided these enzymes into families, based on the catalytic modules in their respective amino acid sequence. Several studies suggest that this database might be a robust tool, affording insights into the carbohydrate-degrading aptitude of a given fungal species and supporting the classification and functional prediction of novel genes [2,4,5]. The CAZy database also enables predicting Auxiliary Activity (AA) families involving lignin-modifying enzymes such as laccases (AA1) and ligninolytic peroxidases (lignin peroxidase, manganese peroxidases and versatile peroxidases) (AA2) [6].

Several members belonging to the *Aspergillus* genus have a widely recognized capacity to degrade lignocellulose [1,2] and, in the last two decades, a growing number of studies have reported on the presence of lignin-modifying enzymes in *Aspergillus* species [7,8].

In recent decades, the significant advances in molecular taxonomy have enabled the discovery of an ever-increasing number of species belonging to the *Aspergillus* genus [9,10], and extreme habitats have offered interesting isolation niches [11,12]. Recently, table olive brine, a liquid matrix commonly hosting lactic acid bacteria and yeast species capable of tolerating high salinity, offered the opportunity to isolate a new species which, due to the isolation source, was denominated *A. olivimuriae* [13]. Although its whole genome has been sequenced, and the isolate is available from two public culture collections, NRRL 66783 and CCF 6208, this species' physiological information is still scant.

To fill this information gap, this study used a combination of bioinformatics and physiological approaches to predict and evaluate, at different levels, the lignocellulose-degrading potential of *A. olivimuriae*. Since most of the biochemical characterization of members of the CAZy families relies on synthetic (model) substrates [5], the use of complex lignocellulosic matrices might be useful to characterize the enzymatic potential of fungi, considering the large variability in the substrate specificity of polysaccharide-degrading enzymes.

Consequently, the profiles of the glycosyl hydrolases and lignin-modifying enzyme activities of *A. olivimuriae* were determined in solid-state fermentation, a culture technique that simulates the conditions that saprophytic organisms encounter in nature. These fermentations were carried out on waste from different supply chains, including residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction (DSF) of anaerobic bioconversions and barley straw. These matrices share the presence of a significant fraction of macromolecules of the plant cell wall (cellulose, hemicellulose and lignin), a characteristic that makes these matrices ideal growth substrates for the expression of enzymes involved in the degradation of these polymers [14,15]. The determination of fungal growth on the substrates under study and the chemical composition changes in the colonized growth substrates offered the necessary integration between genomic and enzymatic analyses.

2. Materials and Methods

2.1. Microorganism and Materials

The fungal strain used in the present study was *Aspergillus olivimuriae* 6C2 (NRRL 66783). The strain was maintained and routinely sub-cultured every month on Malt Extract Agar (MEA) slants. The waste residues used in this work, from four different supply chains, were: residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction (DSF), and barley straw. Expressly, SCG, kindly provided by Prof. Andrea Colantoni (University of Tuscia, Viterbo, Italy), came from the Gedap company (Viterbo, Italy), which deals with automatic coffee distribution. DSF derived from an anaerobic digestion plant, used for biogas production, located near Perugia. RTB, coming from the biorefinery of Porto Torres (Sassari, Italy), is represented by the calathide-deprived epiphytic biomass, from whose seeds the Novamont company extracts dicarboxylic acids used in the production of plasticizers. The barley straw was a kind gift from a farm located in the municipality of Gallese (Viterbo, Italy).

2.2. Annotation of Protein-Coding Genes for Holocellulose Degrading Enzymes

To ascertain the presence of putatively coding genes for carbohydrate-active enzymes (CAZymes) and ligninolytic ones, the genome of *A. olivimuriae* 6C2 (Accession number PRJNA498048) was annotated through the dbCAN2 meta-server (http://bcb.unl.edu/dbCAN2/; accessed on 8 June 2021) which integrates three tools for the annotation of all CAZymes of a genome: (i) HMMER search against the annotated CAZyme domain boundaries according to the dbCAN HMM (hidden Markov model) database (E-value < $1e^{-15}$ and coverage > 0.35); (ii) DIAMOND search for blast hits in the CAZy pre-annotated CAZyme

sequence database (E-value < 1.0×10^{-102}); (iii) Hotpep search for short conserved motifs against the conserved CAZyme short peptide database (hits > 6 and the sum of conserved peptide frequencies > 2.6). The three outputs were combined, and the CAZymes found by only one tool were removed to improve the CAZome annotation accuracy [16]. The output generated by the dbCAN2 meta-server is shown in the Supplementary Materials titled "S1_dbCAN2_output_file_A_olivimuriae". Moreover, to avoid misannotated genes a supplementary manual annotation was carried out, as described by Segato et al. [17]. The Supplementary Materials titled "S2_Annotations" contains the details related to the manual annotation.

2.3. Inoculum Preparation

Six agar plugs (~7 mm Ø) from 7-d-old cultures on MEA were transferred into 500-cm^3 Erlenmeyer flasks containing 100 cm^3 of autoclave-sterilized (394.14 K, 15 min) malt extract broth (Oxoid, Basingstoke, UK) and then incubated at 301.15 K under orbital shaking (2.5 Hz) for 72 h. The mycelium was recovered by centrifugation at 277.15 K (6000× g, 15 min), and the pellet thus obtained suspended in physiological solution. The resulting suspension was then homogenized with an Ultra-Turrax T-18 (IKA Labortechnik, Staufen, Germany) at 50 Hz for 6 s.

2.4. Solid-State Cultures

Each substrate (20 g) was transferred into a 500-cm^3 Erlenmeyer flask, and its moisture content adjusted to a mass fraction (w_m) of 0.70 using the Mandels and Sternberg's medium (MSM), except for SCG, the moisture content of which was adjusted to a w_m equal to 0.55. The composition of MSM was (g dm^{-3}): peptone, 1.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0, urea, 0.3; $CaCl_2$; $MgSO_4 \bullet 7\ H_2O$, 0.3; $FeSO_4 \bullet 7\ H_2O$, 0.005; $MnSO_4 \bullet H_2O$, 0.002; $ZnSO_4 \bullet 7\ H_2O$, 0.001, and $COCl_2$, 0.002. Moreover, an additional set of amended cultures was prepared by adding wheat bran (WB) to each substrate to yield a mass fraction (w_{WB}) equal to 0.20. Each non-amended and amended culture was inoculated with the homogenized inoculum (5 cm^3) to yield an inoculum density of 4.5×10^{-3} g mycelium g^{-1} of solid substrate and incubated in the dark at 301.15 K for 14 d under stationary conditions. For each amended and non-amended culture, a non-inoculated control was prepared and incubated as above. Each experiment was performed in duplicate.

2.5. Analytical Methods

Before chemical analyses, all the materials underwent grinding on a Cyclotec 1093 mill (Foss Analytics, Hilleroed, Denmark) endowed with a 1-mm sieve to make their granulometry uniform. The determination of the crude lipids was carried out gravimetrically after extracting the solid culture samples and the relative incubation controls (3.0 g) in the Büchi B-811 automated Soxhlet apparatus (Uster, Switzerland) with petroleum ether (boiling range 313-333 K) for 6 h, as described by Cruz et al. [18]. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined on defatted samples in a Fibertec System M 1020 Tecator (Foss Analytics, Hilleroed, Denmark) using the method of Van Soest et al. [19]. Hemicellulose was calculated as NDF-ADF and cellulose as ADF-ADL [20], while the ash content in ADL was subtracted from ADL to calculate Klason lignin. Total phenols were determined both on the liquid derived from the hydraulic pressing of solid-state cultures and the resulting residual solid. In the latter case, the material underwent extraction for 4 h at 323 K with a water:methanol mixture, at a volume fraction of 0.50, using a solid:liquid ratio of 0.1 g cm^{-3}, before being analyzed with the procedure of Ainsworth and Gillespie [21], using catechol as the standard. The determination of reducing sugars relied on a 3,5-dinitrosalicylic acid reagent [22]. The elemental analyzer for solids Vario MacroCube of Elementar Gmbh (Langelselbold, Germany) enabled the determination of the percentage contents in carbon, hydrogen, nitrogen and sulfur and the calibration of the measurements relied on sulfonamide, used as the standard. The ash content was determined after the ignition of samples in a muffle furnace at 823 K

for 8 h. The calculation of the organic matter losses (OML, %) relied on the variations in the ash content recorded in the solid phase cultures (x_{ssc}) compared to the respective incubation controls (x_{ic}) according to Equation (1), suggested by Paredes et al. [23]

$$\text{OML } (\%) = 100 - \left[\frac{x_{ic} \times (100 - x_{ssc})}{x_{ssc} \times (100 - x_{ic})} \times 100 \right] \quad (1)$$

The degree of colonization in solid-state cultures was inferred from both visual inspections and indirectly through a fungal-specific marker represented by D, a fungal wall polymers component. In particular, acid hydrolysis and deacetylation of the colonized material relied on the procedure described by Scotti et al. [24], except for the extract's neutralization step, which used solid sodium carbonate. D-glucosamine was spectrophotometrically determined at 530 nm, as reported elsewhere [25]. The same approach was applied to incubation controls to assess the possible presence of D-glucosamine in solid substrates. Soluble protein was determined in the crude extracts derived from the hydraulic pressing of both solid-state cultures and respective incubation controls by the dye-binding method, using bovine serum albumin as the standard [26]. Soluble protein contents in incubation controls were subtracted from those found in the respective solid-state cultures.

2.6. Recovery of the Enzymatic Extract from Solid-State Cultures

At the end of the incubation, 50 cm^3 of sodium acetate buffer (0.01 mol dm^{-3}) at pH 5.0 were added to each culture, and the system was incubated under orbital shaking (3.0 Hz) for 20 min on ice. Except for cultures on spent coffee grounds, in which centrifugation at 277.15 K (8000× g, 20 min) followed the contact time, the other biomasses were subjected to hydraulic pressing to extract the extracellular enzymes. For this purpose, the material was loaded into an extraction cell (15 cm × 4.0 cm i.d.), and fluids were extracted at 0.68 MPa and 277.15 K using a PS-10 press (Ravaglioli, Bologna, Italy). Following extraction, all the extracts underwent sequential filtration through Whatman paper n. 41 and centrifugation at 277.15 K (8000× g, 20 min). The alcohol dehydrogenase activity was measured on the obtained extracts as a cytoplasmic marker to verify possible hyphal damage under the extraction conditions, as reported elsewhere [27].

2.7. Enzymatic Assays

Filter Paper Units (FPU) and endo-β-1,4-glucanase were determined by the method of Ghose [28] using Whatman n. 1 paper and 10 g dm^{-3} carboxymethylcellulose (CMC) solution in sodium acetate buffer (0.05 mol dm^{-3}) at pH 5.0, respectively. At the end of the incubation at 323.15 K, 30 min and 60 min for the former and the latter substrate, Miller's method [22] determined released the reducing sugars. Cellobiohydrolase, endo-β-1,4-xylanase and β-mannanase activities were determined by incubating appropriate volumes of extracts in 10 g dm^{-3} Avicel, 3.0 g dm^{-3}, birchwood xylan and 5.0 g dm^{-3} carob galactomannan solutions at 323.15 K for 30 min, in 0.05 mol dm^{-3} sodium acetate buffer pH 5.0, respectively, and determining the amounts of liberated reducing sugars [29]. β-glucosidase, β-xylosidase and β-mannosidase activities were determined by the method of Meijer et al. [30], except for the volume of the reaction mixtures (1.0 cm^3 in place of 0.1 cm^3) and incubation times, which varied in this study between (15 and 30) min. Polymethylgalacturonase (PMG) and polygalacturonase (PG) activities were determined as described by the method of Heerd et al. [31]. Pectinesterase activity was determined by the method of Vilariño et al. (1993) [32]. Laccase and Mn-peroxidase activities were determined using a modification of the method of del Pilar Castillo et al. [33]. The assay relies on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) promoted by some phenoloxidizing enzymes, leading to a purple chromophore which strongly absorbs in the red region. The reaction mixture used to determine laccase activity contained 7.0 × 10^{-5} mol dm^{-3} MBTH and 1.0 × 10^{-3} mol dm^{-3} DMAB in malonate buffer (0.05 mol dm^{-3}) pH 5.0 and an adequate volume of extract. In the peroxidase assay, the same mixture contained hydrogen peroxide

at a final concentration equal to 2.0×10^{-4} mol dm^{-3}. The absorbance at 590 nm of the reaction mixture was measured immediately downstream of the addition of the enzyme and after predetermined periods of incubation at 323.15 K by calculating the variation in optical density per minute (ODM), and data were converted using an extinction coefficient molar equal to 53,000 dm^3 mol^{-1} cm^{-1}. The peroxidase activity was calculated by subtracting the ODM in the laccase mixture from that measured in the presence of H_2O_2. Regardless of the assay, incubation controls were run in parallel with heat-denatured extracts. Volumetric activities, originally expressed in International Units (IU), defined as the amount of enzyme releasing 1 µmole of product per cm^3 under the assay conditions, were converted into specific activities, referred to either the unit mass of the solid substrate or soluble protein.

2.8. Statistical Analysis

Data were analyzed by one-way ANOVA and post-hoc Tukey test at a significance level of 0.05, using the SigmaStat 3 software (Jandel Corp, San Rafael, CA, USA).

3. Results and Discussion

3.1. Chemical Characterization of the Residues

The first phase of the work focused on characterizing the chemical composition of the four residues and the wheat bran (WB) used in some solid-state cultures as the additive.

The choice of WB was made due to the inclusion, in this investigation, of spent coffee grounds (SCG), a matrix that, owing to its constituent particles' fine granulometry, has low porosity, making gas exchanges problematic. Due to its coarse texture, wheat bran is well-suited to act as a bulking agent capable of increasing the material's overall porosity. Added to this is the fact that the bran has a relatively high nitrogen content (30.1 g kg^{-1}) (Table 1), being mainly composed of protein and amino acids [34]. Furthermore, WB contains microelements and cofactors which are essential for growth, such as B vitamins and folates [35]. Consequently, this study set up and analyzed solid-state cultures on both non-amended and WB-amended residues to make the experimental design homogeneous.

Table 1. Chemical composition of residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction (DSF), barley straw and wheat bran (WB). The data, expressed as g kg^{-1} of the dry weight, are the mean ± standard deviation of 3 determinations. Multiple pair-wise comparisons were made between the row means using the Tukey test; identical letters denote that row means were not significantly different ($p > 0.05$).

Parameter (g kg^{-1})	RTB	SGC	DSF	Barley Straw	WB †
Hemicellulose	189.2 ± 3.2 a	375.2 ± 16.8 c	172.2 ± 14.9 a	279.3 ± 3.0 b	187.6 ± 12.2 c
Cellulose	405.9 ± 12.1 c	120.2 ± 3.2 a	393.4 ± 24.4 c	420.6 ± 2.2 c	201.1 ± 24.3 b
Klason lignin	204.2 ± 4.6 c	201.7 ± 7.2 c	257.4 ± 14.2 d	165.2 ± 4.6 b	73.2 ± 3.9 a
Crude lipids	20.8 ± 1.4 a	139.8 ± 5.4 c	19.3 ± 2.7 a	16.2 ± 1.8 a	45.4 ± 4.3 b
Reducing sugars	3.6 ± 0.4 a	4.1 ± 0.2 a	9.2 ± 0.4 b	41.8 ± 2.7 c	49.3 ± 3.2 d
Phenols	17.2 ± 1.5 b	49.2 ± 2.4 d	15.3 ± 2.4 b	21.4 ± 1.6 c	9.2 ± 1.7 a
Ash	85.3 ± 7.0 d	20.4 ± 2.5 a	82.1 ± 3.6 d	70.1 ± 5.3 c	43.6 ± 2.1 b
Nitrogen	5.9 ± 0.3 a	22.8 ± 1.6 c	19.8 ± 1.1 b	5.4 ± 0.4 a	30.1 ± 1 d
Carbon	423.4 ± 8.0 ab	508.9 ± 11.1 d	415.3 ± 8.2 a	467.4 ± 5.4 c	441.2 ± 4.2 b
Hydrogen	62.1 ± 2.0 b	75.6 ± 1.8 c	59.4 ± 2.1 a	55.2 ± 4.1 a	69.3 ± 4.2 c
Sulphur	1.7 ± 0 b	1.0 ± 0 a	2.2 ± 0.1 b	0.1 ± 0.0 a	1.8 ± 0.0 b

† Starch, the content of which was 175.4 g kg^{-1} in WB, was omitted in this table since this parameter was not detected in the other waste.

Table 1 shows that the holocellulosic component, determined by the cellulose and hemicellulose contents' aggregation, constituted a significant fraction of the residues under study, since its mass fraction (w_{HC}) reached values equal to or well above 0.50 of the dry weight. In particular, the highest w_{HC} was found in barley straw, where the aggregate sum of the cellulose and hemicellulose concentrations gave a value equal to about 0.70, followed by RTB, in which the value of this parameter was slightly less than 0.60. Cellulose was the dominant polysaccharide in RTB, barley straw and DSF (405.9 ± 12.3, 420.6 ± 2.2 and 395.4 ± 24 g kg^{-1}, respectively). Conversely, SCG exhibited the lowest content of this

parameter (120.2 ± 3.2 g kg^{-1}), and hemicelluloses were the most abundant polysaccharide fraction (375 ± 16.8 g kg^{-1}). The monomeric composition of the hemicelluloses in this matrix consists mainly of mannose, galactose and, to a lesser extent, arabinose residues forming galactomannans and arabinogalactans [36]. Moreover, xylose polymers are absent from this matrix, and this is a particular element that differentiates SCG from the remaining residues [36,37]. In addition to its relatively high hemicellulose content, three additional parameters significantly differentiated SCG from other residues in terms of composition. These were the lower ash content (20.4 ± 2.5 g kg^{-1}) and higher crude lipids (139.8 ± 5.4 g kg^{-1}) and total phenols (49.2 ± 2.4 g kg^{-1}) contents than the other three residues. Crude lipid contents in SCG are very similar to those reported in various studies conducted on this matrix [18,38,39]. Concerning the phenolic content in SGC, some studies report concentrations ranging from (17 to 50) g kg^{-1} [18,40], and the main components of this fraction fall within the family of chlorogenic acids within which the quantitatively most relevant compound is 5-O-caffeoylquinic acid [39]. Among the matrices investigated, the lowest phenolic content was in wheat bran (9.2 ± 1.7 g kg^{-1}), although it is considered a relevant source of hydroxy-substituted cinnamic acids [41,42]. However, a significant fraction of these aromatic acids is esterified to the side chains of arabinose residues in arabinoxylans [35] and, therefore, not present in its free form in WB.

Regarding other WB composition data, Table 1 shows that its holocellulosic fraction amounted, overall, to around 390 g kg^{-1}, in agreement with other works [43,44]. For the same matrix, however, the obtained Klason lignin content (73.2 ± 3.9 g kg^{-1}) was significantly lower than those reported by Merali et al. [44] and Prinsen et al. [45], and equal to (108 and 121) g kg^{-1}, respectively.

The Klason lignin contents in RTB and SCG were not significantly different from each other, providing values of about 200 g kg^{-1} while, in barley straw, this parameter amounted to 165.2 ± 2.4 g kg^{-1}. This parameter's highest value, equal to 257 g kg^{-1}, was found in DSF, since stoned olive pomace represents a significant fraction of the feed biomass to the digester from which this matrix derives.

In SCG, the data relating to the total nitrogen content (22.8 g kg^{-1}) and the C/N ratio (22.3), determined by elemental analysis, are also in agreement with a survey conducted by Cruz et al. [18] on a large variety of spent coffee grounds. From this perspective, the C/N ratios of spent coffee grounds and DSF did not significantly differ. This parameter's highest value, equal to about 86.6, was found in barley straw while, in RTB, it was equal to 71.8.

3.2. Fungal Colonization of Solid Substrates and Impact on Their Chemical Composition

One of the significant constraints inherent in solid-state bioconversions, compared to those in the liquid phase, is represented by the impossibility of directly determining the microbial biomass [46]. Contrary to the submerged fermentation, in which the fungal biomass is easily separated from the culture medium and determined by gravimetry, this approach is not possible in solid-state conversions on natural substrates. Therefore, it is necessary to resort to indirect approaches based on either molecular or chemical markers [46]. The most used chemical "markers" for fungi are ergosterol and D-glucosamine. In the first case, the solid material is subjected to saponification, followed by extraction with n-hexane and quantification of ergosterol by reverse-phase chromatography [20]. The second parameter's determination requires acid hydrolysis of the material, deacetylation, and quantification of the D-glucosamine, released by chromatography or spectrophotometry [24]. However, D-glucosamine levels vary in fungal species in a range of from 67 to 125 mg g^{-1} of biomass, depending on the culture's species and age [14,47]. Although aware that cultivation conditions can also influence this parameter's levels, we adopted D-glucosamine as an indirect indicator of colonization levels, bearing in mind that the comparisons took place within the same fungal species and between cultures of the same age. We also considered the possible presence of D-glucosamine residues which are sometimes associated with glycoproteins in the lignocellulosic substrates [14]. Although this parameter's levels were significantly low

in the incubation controls, varying in the range of from 0.03 to 0.18 mg g^{-1} substrate (data not shown), these were subtracted from those determined in solid-state cultures.

Table 2 shows that, among the non-amended cultures, the lowest growth was observed on barley straw, while the colonization levels did not differ significantly from each other in the remaining substrates, ranging from 0.79 to 0.94 mg D-glucosamine g^{-1} substrate. On the contrary, an element that emerged from the D-glucosamine data analysis was the stimulating effect of WB on fungal growth in the amended cultures compared to non-amended ones except those carried out on DSF. This stimulation effect was of variable magnitude depending on the substrate. In particular, in solid-state cultures on WB-amended barley straw, the colonization levels increased by a factor of more than four compared to non-amended counterparts (2.99 ± 0.5 vs. 0.70 ± 0.04 mg D-glucosamine g^{-1}), and visual inspections corroborated the observed data. A loose mycelium with a limited extension to the most peripheral layers of the substrate characterized non-amended cultures on barley straw. Conversely, in WB-amended cultures, the mycelium appeared to be more compact, and the colonization affected the entire substrate mass. In WB-amended cultures on RTB and SCG, the factors of increase in D-glucosamine levels compared to homologous non-amended cultures were equal to 2.2 and 2.7, respectively.

Table 2. D-glucosamine and extracellular protein (EXP) contents, pH changes, organic matter losses (OML) and yield of fungal biomass per unit of organic matter consumed ($Y_{GLC/S}$) in 14-day-old *A. olivimuriae* solid-state cultures carried out on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and solid fraction of digestate (SFD) in the presence and in the absence of wheat bran (WB). Data are mean ± standard deviation of 4 replicates. Multiple pair-wise comparisons were made with Tukey's test, and identical letters denote that column means were not statistically different ($p > 0.05$).

Solid-State Cultures On:	D-Glucosamine (mg g^{-1})	EXP (mg g^{-1})	ΔpH †	OML ‡ (%)	$Y_{GLC/S}$ (mg g^{-1})
RTB	0.87 ± 0.05 ab	2.89 ± 0.24 b	−0.80 (6.93 ± 0.03)	12.64 ± 0.40 c	6.88 ± 0.50 b
RTB + WB	1.89 ± 0.07 c	3.49 ± 0.11 c	−0.56 (6.43 ± 0.04)	17.01 ± 0.60 e	11.11 ± 0.41 c
SCG	0.94 ± 0.06 b	2.00 ± 0.09 a	−1.75 (5.77 ± 0.04)	10.77 ± 1.21 b	8.73 ± 0.46 b
SCG + WB	2.47 ± 0.08 d	1.89 ± 0.12 a	−0.73 (5.34 ± 0.02)	14.70 ± 0.81 d	16.80 ± 0.54 d
BS	0.70 ± 0.06 a	5.46 ± 0.33 d	n.s. * (6.50 ± 0.04)	8.43 ± 0.34 a	8.30 ± 1.30 b
BS + WB	2.99 ± 0.21 e	5.04 ± 0.18 d	−0.17 (6.83 ± 0.02)	12.24 ± 0.78 bc	24.44 ± 1.63 e
DSF	0.79 ± 0.04 ab	3.68 ± 0.07 c	+0.24 (6.60 ± 0.05)	17.73 ± 0.51 e	4.46 ± 0.49 a
DSF + WB	0.87 ± 0.03 ab	3.70 ± 0.38 c	+0.64 (6.92 ± 0.08)	17.03 ± 0.46 e	5.11 ± 0.7 ab

(†) The data in round brackets are the pH values found in the non-inoculated incubation controls; (‡) calculated from the ash content in solid-state cultures and related incubation controls using the equation of Paredes et al. (2002); * n. s., not significant (pH values of solid-state cultures did not significantly differ from the respective incubation controls).

In *A. olivimuriae* solid-state cultures, colonization led, compared to incubation controls, to the slight acidification of some matrices, such as in RTB (6.13 ± 0.02 vs. 6.93 ± 0.03) and, to a greater extent, in SCG (4.02 ± 0.05 vs. 5.77 ± 0.04), and this effect was also observed in the respective WB-amended cultures. In those carried out on barley straw, the pH values did not differ significantly from those of the relative incubation control while, on the WB-amended ones, slight acidification took place (6.66 ± 0.02 vs. 6.88 ± 0.01). Although not experimentally demonstrated, the acidification observed in solid-state cultures was presumably due to the production of organic acids, a general metabolic characteristic among the species belonging to the *Aspergillus* genus that can lead to the substrate pH being lowered by four units [14,48,49].

The production of organic acids by these species seems to be a strategy implemented in nature to counteract the antagonistic effect exerted by bacteria [50]. Conversely, on the DSF, the solid-state cultures' pH values were higher than those of the incubation controls, albeit with limited variations. The lack of acidification on this matrix may be due to the known buffering capacity of the digestate [51].

Table 2 also shows the biotic losses of organic matter are attributable to mineralization and calculated based on the ash contents of the solid-phase cultures and the related

incubation controls. The amendment's presence increased the extents of organic matter losses in thistle biomass, barley straw and SCG. Conversely, this parameter's values did not significantly differ in non-amended and amended cultures on DSF (17.73 ± 0.51 and 17.03 ± 0.46, respectively). The values of the biomass yields ($Y_{GLC/S}$), derived by relating D-glucosamine levels to the unit mass of organic matter consumed, did not significantly differ in non-supplemented cultures, while the presence of the WB markedly increased this parameter in amended ones.

Table 3 shows the contents in cellulose, hemicellulose, lignin, reducing sugars, crude lipids and total phenols in the incubation controls and the respective cultures inoculated with *A. olivimuriae*. ANOVA was conducted on four homogeneous blocks represented by each substrate to verify the significance of the differences between each parameter's values in the cultures and the incubation controls in both the amended and non-amended cultures. Regarding the changes induced by the fungus on the cell wall polysaccharide contents, the percentage removal values of the hemicelluloses were significantly higher than those of cellulose, with the only exception being represented by cultures grown on WB-amended RTB; under these conditions, cellulose removal significantly increased compared to homologous non-amended cultures (23.5 vs. 12.7%). The percentage of hemicellulose removal varied widely, ranging from 12% in cultures grown on SCG to 59.7% in non-amended DSF ones. WB's general effect in amended cultures was to determine an increase in wall polysaccharides' removal levels compared to those observed in non-amended ones. RTB and DSF were exceptions to this rule, since non-amended cultures outperformed amended ones in hemicellulose removals (34 vs. 26.7% and 59.7 vs. 47.6%, respectively). The presence of WB generally improved the cellulose removal in the amended cultures, even though, in some cases, the extents of these increases were slight. In general, this study observed a higher susceptibility of hemicelluloses to degradation than cellulose, regardless of the substrate and presence or absence of WB. These results are in line with several studies which ascribe the higher recalcitrance of cellulose to biodegradation rather than hemicellulose, due to its partial crystallinity [14,52].

The increase in reducing sugars observed on solid-state cultures on RTB and SCG, compared to the respective incubation controls, testifies to the partial degradation of the structural polysaccharides, although the concentrations of reducing sugars are the result of the balance between the hydrolysis of the polymers themselves and fungal uptake of the monosaccharides made available by the hydrolysis itself. In these cultures, comparing the yield in reducing sugars released by the hydrolysis of cellulose and hemicelluloses and those found in the extracts, it is evident that *A. olivimuriae* metabolized a high fraction of these (data not shown).

Table 3 shows that, with few exceptions, the Klason lignin contents in the cultures did not differ significantly from their respective incubation controls, apparently suggesting that *A. olivimuriae* does not have ligninolytic aptitudes, as was widely thought for several years for most *Aspergillus* species [53]. Some studies, conducted in solid-state fermentation and simultaneous saccharification and fermentation with other organisms, suggest that the preferential degradation of cellulose and hemicellulose inevitably leads to a shift in the relative percentage proportions of the wall macromolecules, thus leading to a lignin-enriched residue [20,54]. For this reason, some studies have proposed normalizing the lignin mass fractions to their respective ash contents [20,23]. However, even after this normalization, no significant lignin removal was evident in *A. olivimuriae* solid-state cultures. In this respect, this species differs from other Aspergilli, such as *A. oryzae* [8] and *A. flavus* [55], which have shown ligninolytic potential.

Except for the non-amended cultures grown on DSF and barley straw, total phenol concentrations were significantly higher than those of the respective incubation controls. To explain these results, we could hypothesize the hydrolysis of glucosides by β-glucosidase, leading to the release of phenolic aglycones. Alternatively, we might invoke a partial lignin fragmentation, not translating into a substantial decrease in this parameter but leading to

the liberation of phenolic hydroxyls or the release of hydroxycinnamic acids from WB in amended cultures due to the action of *A. olivimuriae* feruloyl esterase.

Table 3. Contents of cellulose, hemicellulose, Klason lignin, reducing sugars, total phenols and crude lipids in non-inoculated incubation controls (IC) and 14-day-old *A. olivimuriae* solid-state cultures (SSC) on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and digestate solid fraction (DSF) in the absence and in the presence of wheat bran (WB). Data represent the mean ± standard deviation of 4 replicates. Multiple pairwise comparisons were carried out within each matrix using the Tukey test, and identical letters denote that column means within each block were not significantly different ($p > 0.05$).

Culture	Cellulose (g kg^{-1})	Hemicellulose (g kg^{-1})	Klason Lignin (g kg^{-1})	Reducing Sugars (g kg^{-1})	Crude Lipids (g kg^{-1})	Total Phenols (g kg^{-1})
RTB (IC)	405.6 ± 12.1 c	187.0 ± 3.3 c	201.1 ± 8.0 a	3.3 ± 0.6 a	20.9 ± 2.0 b	17.3 ± 2.3 a
RTB (SSC)	354.2 ± 4.0 b	121.9 ± 4.4 a	217.5 ± 2.2 a	17.7 ± 2.2 b	14.5 ± 1.3 a	38.3 ± 4.2 c
RTB + WB (IC)	365.6 ± 13.4 b	167.7 ± 6.2 b	181.2 ± 14.4 a	3.7 ± 0.5 a	26.5 ± 2.1 c	15.6 ± 3.4 a
RTB + WB (SSC)	279.6 ± 16.3 a	123.4 ± 12.1 a	204.8 ± 19.5 a	16.4 ± 0.6 b	15.3 ± 3.2 a	23.8 ± 2.8 b
SCG (IC)	119.2 ± 3.0 b	370.2 ± 16.3 c	199.4 ± 18.5 b	3.2 ± 0.0 a	139.0 ± 6.4 c	48.7 ± 3.2 b
SCG (SSC)	105.4 ± 4.4 a	326.3 ± 18.3 b	240.0 ± 21.3 c	7.6 ± 1.1 b	109.2 ± 4.1 b	56.0 ± 2.3 c
SCG + WB (IC)	136.2 ± 3.2 c	337.1 ± 8.6 bc	171.4 ± 5.2 a	3.1 ± 0.5 a	121.2 ± 8.2 b	40.8 ± 2.1 a
SCG + CG (SSC)	103.5 ± 3.1 a	255.4 ± 21.6 a	176.5 ± 5.2 ab	9.6 ± 0.4 c	75.8 ± 10.3 a	51.5 ± 2.6 bc
BS (IC)	420.1 ± 3.0 d	279.5 ± 11.2 c	165.0 ± 14.3 a	41.3 ± 2.8 a	15.6 ± 1.1 a	20.7 ± 2.1 ab
BS (SSC)	393.9 ± 3.2 c	237.0 ± 9.3 b	172.6 ± 8.2 a	51.3 ± 2.0 b	12.0 ± 1.9 a	27.1 ± 4.3 b
BS + WB (IC)	376.1 ± 8.3 b	262.7 ± 8.3 c	159.0 ± 14.8 a	43.4 ± 4.6 a	21.4 ± 2.4 b	18.4 ± 4.2 a
BS + WB (SSC)	339.7 ± 5.0 a	210.4 ± 7.6 a	170.6 ± 13.3 a	40.8 ± 5.4 a	20.3 ± 3.0 b	25.4 ± 3.0 b
DSF (IC)	393.2 ± 4.6 c	174.1 ± 12.3 c	257.2 ± 11.6 bc	10.9 ± 0.5 b	19.2 ± 3.1 b	15.6 ± 1.6 b
DSF (SSC)	363.0 ± 7.1 b	70.2 ± 9.1 a	272.0 ± 8.3 c	4.6 ± 0.3 a	21.4 ± 0.2 b	11.9 ± 2.0 a
DSF + WB (IC)	354.2 ± 4.1 b	177.3 ± 8.6 c	220.3 ± 4.8 a	17.4 ± 3.1 c	22.4 ± 3.1 b	14.3 ± 1.3 ab
DSF + WB (SSC)	321.3 ± 12.0 a	92.7 ± 8.3 b	236.1 ± 5.2 b	4.9 ± 0.1 a	4.4 ± 0.2 a	12.9 ± 1.1 a

Crude lipids were minor components in RTB, barley straw, and DSF, reaching values in the incubation controls lower or slightly higher than 20 g kg^{-1}. The only exceptions were represented by the uncorrected and amended SCG incubation controls, where this parameter amounted to around (139 and 121) g kg^{-1}, undergoing a reduction of 24 and 37.3%, respectively, in solid-state cultures.

3.3. Gene Prediction of Holocellulose Degrading Enzymes in A. olivimuriae

Genomes of *Aspergillus* species code for a high number of CAZymes involved in the degradation of plant polysaccharides, including cellulose, hemicellulose (xylan, xyloglucan, arabinoxylan and glucomannan) and pectin. Several glycosyl hydrolases (GH) families have been characterized and predicted in Aspergilli, along with carbohydrate esterases (CE) and polysaccharide lyases (PL) [3].

Different *Aspergillus* species (e.g., *A. niger*, *A. fumigatus*, *A. tamarii*, *A. nidulans*) showed the occurrence of genes coding for lignin-modifying enzymes [3,56], classified in the family of auxiliary activity enzymes (AA). These include multi-copper oxidases, such as laccases, ferroxidases and laccase-like multi-copper (AA1), heme peroxidases, such as lignin peroxidases, manganese peroxidases and versatile peroxidases (AA2) and, finally, 1,4-benzoquinone reductases (AA6).

Recently, lytic polysaccharide monooxygenases (LPMO) have also received special attention due to their boosting effect on hydrolytic enzymes' activity, suggesting a fundamental role of redox reactions in lignocellulose conversion. In the CAZy database, LPMOs belong to six AA families: AA9, AA10, AA11, AA13, AA14 and AA15, among which, the most widely studied are AA9 and AA10 [57].

In *A. olivimuriae*, the genome annotation of CAZymes allowed identifying a total of 148 protein-coding genes involved in lignocellulose degradation, belonging to 42 families. Among them, 109 genes were assigned to GH, 6 to CE, 3 to PL and 30 to AA. CAZymes were further subdivided based on their substrate and the family they belong to, as suggested

by Lombard et al. [58], and compared to *Aspergillus* species already characterized for their CAZyme content (Table 4) [3].

Table 4. Comparison of predicted number of glycosyl hydrolases (GH), carbohydrate esterases (CE), polysaccharide lyases (PL) and of auxiliary activities (AA) found in the genome of *A. olivimuriae* (*Aol*) (this work), *A. fumigatus* (*Af*), *A. nidulans* (*And*), *A. niger* (*Ang*) and *A. oryzae* (*Aor*).

Substrate		Enzyme Activity	CAZyme Families	Number of Protein-Coding Predicted Genes				
				Aol †	*Af* [a]	*And* [a]	*Ang* [a]	*Aor* [a]
Cellulose		β-1,4-Endoglucanase	GH3, -5, -6, -7, -9, -12, -45	35 (22)	41	42	36	41
		Cellobiohydrolase	GH7	3 (3)	4	3	2	3
		β-1,4-Glucosidase	GH1, -3	17 (11)	23	23	22	26
		Cellobiose dehydrogenase	AA3, AA8	2 (2)	2	2	2	2
		Lytic polysaccharide monooxygenase	AA9	6 (6)	7	9	8	8
Hemicellulose	Xylan	β-1,4-Endoxylanase	GH10, -11, -30	6 (5)	8	5	6	8
		β-1,4-Xylosidase	GH3, -43	30 (15)	36	35	30	43
	Galactomannan	β-1,4-Endomannanase	GH5	15 (5)	13	15	10	13
		β-1,4-Mannosidase	GH2	6 (2)	6	10	6	7
		β-1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
		α-1,4-Galactosidase	GH27, -36	3 (2)	3	4	2	3
		α-Arabinofuranosidase	GH51, -54	3 (3)	3	3	5	4
	Xyloglucan	β-1,4-endoglucanase	GH12, -74	3 (3)	6	3	4	4
		α-, β-Arabinofuranosidase	GH51, -54, -127	2 (3)	3	4	2	3
		α-Xylosidase	GH31	10 (1)	n.a.	10 [b]	7 [b]	10 [b]
		α-Fucosidase	GH29, -95	2 (0)	n.a.	3 [b]	3 [b]	3 [b]
		α-1,4-Galactosidase	GH27, -36	3 (2)	3	4	2	3
		β -1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
	Arabinoxylan	Arabinofuranohydrolase	GH62	2 (2)	2	2	1	2
		α-, β-Glucuronidase	GH67, -115, -154	4 (2)	2	2	1	5
		α-1,4-Galactosidase	GH27, -36	3 (2)	3	4	2	3
		β-1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
		Acetyl xylan esterase	CE1, -5	3 (4)	10	7	8	10
		Feruloyl esterase	CE1	1 (1)	5	3	3	5
Pectin		Endo-polygalacturonases	GH28	3 (1)	14	9	22	21
		α-Rhamnosidase	GH78, 106	7 (0)	6	8	8	9
		α-Arabinofuranosidase, Arabinofuranohydrolase	GH51, -54, -62	4 (5)	5	5	6	6
		Endoarabinanase	GH43	14 (5)	18	15	11	20
		Exoarabinanase	GH93	3 (2)	3	2	1	3
		β -1,4-Endogalactanase	GH53	1 (1)	1	1	1	1
		Unsaturated glucuronyl hydrolase	GH88	1 (0)	n.a.	2 [b]	1 [b]	3 [b]
		Unsaturated rhamnogalacturonan hydrolase	GH105	2 (0)	n.a.	3 [b]	2 [b]	4 [b]
		β-1,4-Xylosidase	GH3, -43	30 (15)	36	35	30	43
		β-1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
		Pectate lyase	PL1	2 (0)	6	8	7	12
		Rhamnogalacturonan lyase	PL4	1 (0)	3	4	2	4
		Pectin methyl esterase PME	CE8	1 (0)	6	3	3	5
		Rhamnogalacturonan acetyl esterase	CE12	2 (0)	3	2	2	4
		Feruloyl esterase CE1	CE1	1 (3)	5	3	3	5
Lignin		Laccase/p-diphenol:oxygen oxidoreductase	AA1	3 (2)	1 [c]	2 [b]	2 [c]	4 [b]
		Lignin-modifying peroxidases	AA2	2 (0)	3 [c]	0 [b]	2 [c]	0 [b]
		1,4-benzoquinone reductase	AA6	1 (1)	0 [c]	0 [b]	2 [c]	1 [b]

[a] Data from [55], integrated with [b] Cazy database (http://www.cazy.org/; accessed on 8 June 2021); [c] data from [3]. † For *A. olivimuriae*, the number of genes that have beeen manually annotated is shown between round brackets; The supplementary material titled "Annotations" contains the details related to the manual annotation.

A significant number of enzymes related to cellulose, hemicellulose and pectin degradation was identified [2,3]. In particular, the number of predicted genes for each family was close to that found in other *Aspergillus* species, except for acetyl xylan esterase and endo-

polygalacturonases, which are significantly lower in number in *A. olivimuriae* (Table 4). The most frequently found glycoside hydrolases were endo β-1,4-glucanase (GH3,GH5, GH6,GH7, GH9, GH12, GH45) followed by β-xylosidase (GH3, GH43).

The number of genes related to lignin-modifying enzymes in *A. olivimuriae* was low and CAZy analysis identified 2, 1 and 3 genes coding for laccase, 1,4-benzoquinone reductase and lignin-modifying peroxidases, which, however, is in line with other *Aspergillus* species [3].

The presence of putative CAZyme-encoding genes in *A. olivimuriae* genome provides insight into its lignocelluloses-degrading enzyme potential, but cannot be directly related to its real degradation ability. In fact, in several works, it has been observed that the number of genes related to the degradation of a given polysaccharide is not necessarily correlated to the extents of its degradation, since fungal species rely on different strategies. For instance, *Trichoderma reesei* relies on the high production levels of a limited set of glycosyl hydrolases, while the white-rot basidiomycete *Phanerochaete chrysosporium* has a large repertoire of these enzymes [56]. For this reason, CAZy analysis is necessarily associated with functional approaches, such as enzymatic activity assays, and wet chemistry methods, to gain valuable insight into the actual behavior of the species of concern on specific lignocelluloses.

3.4. Solid-State Production of A. olivimuriae Extracellular Glycosyl Hydrolases and Lyases

As expected from the composition changes in the colonized materials and the CAZy prediction, we detected both glycosyl-hydrolase and lyase activities involved in the depolymerization of plant cell-wall polysaccharides in *A. olivimuriae* solid-state cultures. However, CAZyme production levels varied depending on the residue used and the presence or absence of the amendment.

Regarding cellulolytic enzymes, non-amended and amended cultures on RTB yielded the highest endo-β-1,4-glucanase activities (4.11 ± 0.81 and 4.40 ± 0.69 IU g^{-1} substrate, respectively) (Figure 1A). The presence of WB significantly increased this activity compared to homologous non-amended ones, but only in cultures on barley straw (4.40 ± 0.58 vs. 3.20 ± 0.59 IU g^{-1} substrate, respectively). Amended and non-amended cultures on RTB exhibited the highest cellobiohydrolase activity, and the presence of WB resulted in a significant increase in this activity, but only for SCG cultures (2.51 ± 0.42 vs. 1.51 ± 0.28 IU g^{-1} substrate) (Figure 1B). As was already observed for endo-β-1,4-glucanase and cellobiohydrolase, among non-amended cultures, those on RTB gave the highest β-glucosidase activity and, in this case, the presence of WB led to a doubling of production levels (2.68 ± 0.38 vs. 1.33 ± 0.15 IU g^{-1} substrate) (Figure 1C). The total cellulolytic activity was also determined using Whatman filter paper n. 1, a substrate with the merit of the inclusion of amorphous and para-crystalline regions, whose use was proposed by Ghose [28] and, later on, recommended by the IUPAC. For this reason, the activity of many commercial cellulolytic preparations is expressed in terms of Filter Paper Units [59]. Figure 1D shows that solid-state cultures on RTB and barley straw yielded the best activity values. The effect of WB was statistically significant in cultures carried out on RTB (2.27 ± 0.40 vs. 1.48 ± 0.34 FPU g^{-1} substrate), barley straw (1.95 ± 0.26 vs. 2.98 ± 0.60 FPU g^{-1} substrate) and in those on DSF (0.97 ± 0.12 vs. 0.47 ± 0.03 FPU g^{-1} substrate) (Figure 1D). Notably, CMCase, FPU and β-glucosidase production levels by *Aspergillus coespitosus* NRRL 1929 and *A. tubingensis* NRRL 4700 solid-state cultures on RTB were very similar to those observed with the strain under study on the same substrate [60]. DSF was poorly conducive to cellulase production in line with Mejias' results [51]; in that study, in the solid-state cultures of *T. reseei* ATCC 26921, a well-known cellulase-producing strain, the enzymatic activity never exceeded 1.1 FPU g^{-1} digestate.

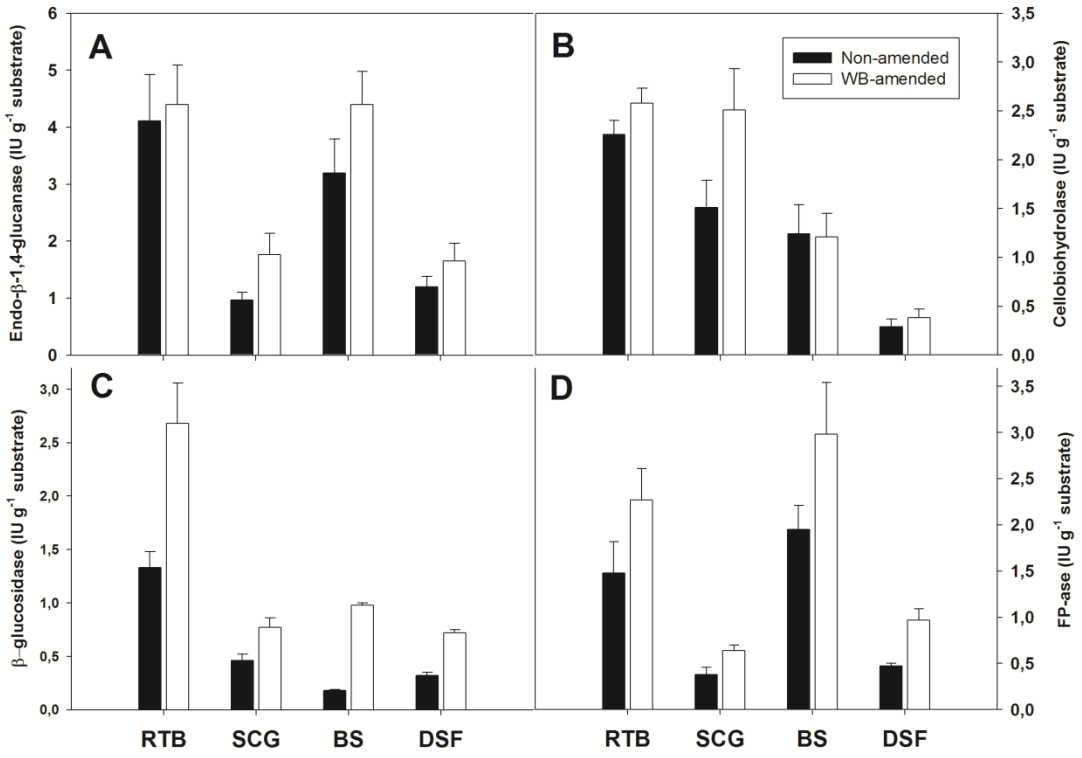

Figure 1. Specific activities, referred to unit mass of initial substrate, of endo-β-1,4-glucanase (**A**), cellobiohydrolase (**B**), β-glucosidase (**C**), and FP-ase (**D**) in 14-day-old solid-state cultures conducted on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and digestate solid fraction (DSF) in the presence and in the absence of wheat bran (WB) as the amendment. Data are the mean ± standard deviation of four replicates.

However, *Aspergillus olivimuriae* 6C2 cellulase production levels do not compare with hyper-producing strains belonging to the same genus. To exemplify, *Aspergillus terreus* M11 solid-state cultures on different substrates, such as rape straw, wheat straw, wheat bran and corn stover, reached enzymatic CMCase and FPU activity levels ranging from 122 to 440 IU g^{-1} and 12 to 198 IU g^{-1}, respectively [61]. CMCase, β-glucosidase and FPU activities in *A. fumigatus* ABK9 grown on WB-rice straw mixed substrate led to 824.21 and 254.44 and 106.2 IU g^{-1}, respectively [62].

Concerning hemicellulases, this study determined the activities of enzymes involved in the hydrolysis of two ubiquitous hemicellulose components, namely xylan and galactomannan. In addition to the endo-β-1,4-xylanase and galactomannase activities, which hydrolyze the β(1 → 4) glycosidic bonds between adjacent residues within the chain and in a random manner, we determined the activities of two exo-enzymes acting on these two polymers, namely β-xylosidase and β-mannosidase. The former catalyzes the xylobiose hydrolysis and the detachment of xylooligosaccharides from the xylan's non-reducing end. The latter brings about the detachment of mannosyl residues from the mannans' non-reducing end.

Among the non-amended cultures, those on barley straw attained the endo-β-1,4-xylanase activity (7.56 ± 0.79 IU g^{-1} substrate), but RTB cultures also led to significant activity levels (5.52 ± 1.17 IU g^{-1} substrate) (Figure 2A). Although the presence of WB increased the activity levels of this enzyme in cultures on barley straw and RTB (8.95 and 6.65 IU g^{-1} substrate, respectively), the only statistically significant increase occurred on SCG (3.23 ± 0.76 vs. 1.04 ± 0.18 IU g^{-1} substrate). It is likely that WB stimulated this enzyme's production, since this amendment brings xylose-based polymers to the growth substrate, whereas the hemicellulose fraction in SCG is devoid of these components [36,39]. On the same growth substrate, the presence of WB also led to a 4.5-fold increase in β-xylosidase activity (Figure 2B). As evidence of the previous considerations

about the hemicellulose composition in SCG, the highest β-mannanase activity was found precisely on this matrix (3.72 ± 0.2 IU g^{-1} substrate) (Figure 2C), thus confirming the adequacy of coffee wastes to support the production of this enzyme by *Aspergillus* species [63]. The β-mannosidase activity was also the highest in cultures carried out on non-amended SCG (0.90 ± 0.04 IU g^{-1} substrate) compared to other cultures (Figure 2D). Regardless of the growth substrate, the amendment's presence did not significantly affect the production of both β-mannanase and β-mannosidase (Figure 2C,D). As was already observed for cellulolytic enzymes, *A. olivimuriae* production levels of hemicellulases on the tested residues were significantly lower than those achieved by solid-state cultures of other *Aspergillus* species on different residues. For instance, de Oliveira Rodrigues et al. [64] found that *A. niger* SCBM1 and *A. fumigatus* SCBM6, grown individually on a mixture of spent bagasse and WB, produced very high activity levels for both endo-β-1,4-xylanase (2271.7 and 1164.8 IU g^{-1} substrate, respectively) and β-xylosidase (145 and 2.5 IU g^{-1} substrate, respectively).

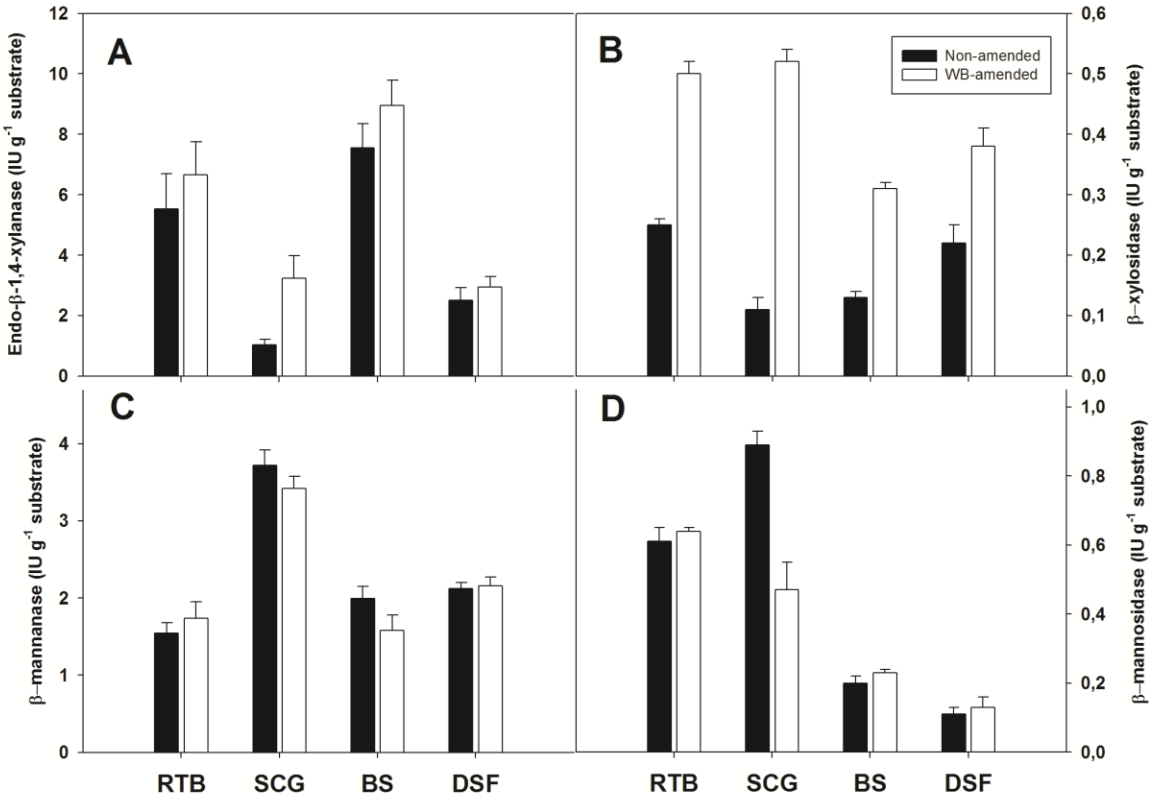

Figure 2. Specific activities, referred to unit mass of initial substrate, of endo-β-1,4-xylanase (**A**), β-xylosidase (**B**), β-mannanase (**C**), and β-mannosidase (**D**) in 14-day-old solid-state cultures conducted on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and digestate solid fraction (DSF) in the presence and absence of wheat bran (WB) as the amendment. Data are the mean ± standard deviation of four replicates.

Although none of the used solid substrates is a pectin-rich material, the investigation was also extended to the determination of polymethylgalacturonase (E.C. 3.2.1.15), polygalacturonase (E.C. 3.2.1.67) and pectinesterase (E.C. 3.1.1.11) activities. Polymethylgalacturonase, ambiguously called pectinase, catalyzes the random hydrolysis of α (1 → 4) bonds between adjacent residues in pectate and other galacturonans and, depending on the microbial source, has different tolerances to the methyl esterification of galacturonic residues. On the contrary, polygalacturonase is specific to D-homogalacturonan oligomers and cannot degrade unsaturated or methyl esterified substrates. The role of pectinesterase is to facilitate the action of depolymerizing enzymes by hydrolyzing the carboxylic esters present in the D-galacturonic residues of the pectin, producing pectates and methanol.

In addition to these hydrolases' activities, the activities of pectin lyase (EC 4.2.2.10) and pectate lyase (E.C. 4.2.2.2) were also measured. These enzymes break the α (1 → 4) bond between D-galacturonic residues adjacent through a β-elimination mechanism that leads to the production at the non-reducing end of one of the two fragments of an unsaturated residue between C4 and C5 (4-deoxy-alpha-D-galact-4-en-uronosyl groups).

Table 5 shows that, among the non-amended substrates, the best polymethylgalacturonase, polygalacturonase and pectinesterase activities were found in cultures carried out on RTB (2.30 ± 0.18, 1.27 ± 0.21 and 1.24 ± 0.20 IU g^{-1} substrate, respectively). Significantly increased polymethylgalacturonase activities, due to the presence of WB compared to the non-amended counterpart, were found on RTB (2.99 ± 0.27 vs. 2.30 ± 0.18 IU g^{-1} substrate) and barley straw (1.49 ± 0.10 vs. 0.99 ± 0.13 IU g^{-1} substrate). In the case of polygalacturonase, the only cultures to benefit from the presence of WB were those carried out RTB (1.93 ± 0.09 vs. 1.27 ± 0.21 IU g^{-1} substrate) and pectinesterase activities (2.16 ± 0.33 vs. 1.24 ± 0.20 IU g^{-1} substrate). Although detected in all the solid-state cultures, the lyase activities were significantly lower than those of hydrolases (Table 5).

Table 5. Specific activities, referred to unit mass of the solid substrate, of polymethylgalacturonase (PMG), pectin esterase (PE), polygalacturonase (PG), pectin lyase, and pectase lyase in *A. olivimuriae* solid-state cultures conducted for 14 days at 301.15 K on residual thistle biomass (RTB), spent coffee rounds (SCG), barley straw (BS) e digestate solid fraction (DSF) in the absence and presence of wheat bran (WB). Data are the means ± standard deviation of 4 replicates. Multiple pair-wise comparisons were made with Tukey's test, and identical letters denote that column means were not statistically different ($p > 0.05$).

Culture	PMG (IU g^{-1} Substrate)	PE (IU g^{-1} Substrate)	PG (IU g^{-1} Substrate)	Pectin Lyase (IU g^{-1} Substrate)	Pectate Lyase (IU g^{-1} Substrate)
RTB	2.30 ± 0.18 c	1.24 ± 0.20 c	1.27 ± 0.21 c	0.33 ± 0.08 bc	0.11 ± 0.03 a
RTB + WB	2.99 ± 0.27 d	2.16 ± 0.33 d	1.93 ± 0.09 d	0.31 ± 0.01 b	0.08 ± 0.01 a
SCG	0.66 ± 0.05 a	0.39 ± 0.03 ab	0.54 ± 0.06 a	0.17 ± 0.05 a	0.12 ± 0.06 ab
SCG + WB	0.73 ± 0.08 a	0.53 ± 0.08 b	0.56 ± 0.11 a	0.15 ± 0.01 a	0.10 ± 0.02 a
BS	0.99 ± 0.13 a	0.10 ± 0.01 a	0.74 ± 0.11 ab	0.19 ± 0.04 a	0.20 ± 0.04 ab
BS + WB	1.49 ± 0.13 b	0.06 ± 0.02 a	0.91 ± 0.11 b	0.31 ± 0.02 b	0.25 ± 0.04 b
DSF	0.49 ± 0.12 a	0.14 ± 0.05 ab	0.52 ± 0.07 a	0.19 ± 0.04 a	0.22 ± 0.02 b
DSF + WB	0.71 ± 0.09 a	0.09 ± 0.02 a	0.73 ± 0.11 a	0.46 ± 0.05 c	0.26 ± 0.04 b

3.5. Solid-State Production of Lignin-Modifying Enzymes by A. olivimuriae

A further purpose of this work was to verify the presence of ligninolytic enzymes in *A. olivimuriae* 6C2 solid-state cultures and determine their activity. This strain, mistakenly identified in a previous study as *A. fumigatus* 6C2, based on ITS sequences and morphological characters, underwent a battery of agar plate tests that indicated the presence of lignin-modifying enzymes (formation of a brown halo in the Bavendamm's test with tannic acid, decolorization of Poly-R478, formation of ABTS oxidation halos using the underlay agar technique) [65]. In the same study, the strain produced detectable laccase and peroxidase activities in the same work, albeit at low levels. Following the correct identification of this species and sequencing of its genome, this study assessed the presence of putative genes coding for ligninolytic enzymes.

Therefore, it was reasonable to expect these enzymes' presence under a cultural condition, such as solid-state fermentation on lignocellulose-based substrates, which reproduces those encountered in natural habitats. As shown in Table 6, activity levels were very low and in line with those found in the previously mentioned work [65]. Among the non-amended cultures, the best laccase and peroxidase production levels were found in RTB and SCG; in these growth substrates, the presence of WB led to significantly increased laccase and peroxidase activities. However, on the tested substrates, the production levels of these enzymes were markedly lower than those observed in solid-state cultures of other *Aspergillus* species, such as *A. fumigatus* SCBM6 and *A. niger* SCBM1 and ATCC 1004 conducted on different residues [64,66]. For instance, solid-state cultures of *A. niger* ATCC

1004, grown on palm cactus husk, yielded laccase activity, which was around 173-fold higher than the best production achieved with *A. olivimuriae* [66].

Table 6. Specific activities, referred to unit mass of the solid substrate and extracellular protein, of laccase and peroxidase in *A. olivimuriae* solid-state cultures conducted for 14 days at 301.15 K on residual thistle biomass (RTB), spent coffee rounds (SCG), barley straw (BS) e digestate solid fraction (DSF) in the absence and in the presence of wheat bran (WB). Data are the means ± standard deviation of 4 replicates. Multiple pair-wise comparisons were made with Tukey's test, and identical letters denote that column means were not statistically different ($p > 0.05$).

Culture	Laccase		Peroxidase	
	(IU g^{-1} Substrate) ($\times 10^{-3}$)	(IU mg^{-1} Protein) ($\times 10^{-3}$)	(IU g^{-1} Substrate) ($\times 10^{-3}$)	(IU mg^{-1} Protein) ($\times 10^{-3}$)
RTB	32.8 ± 0.4 c	11.3 ± 0.1	30.0 ± 1.6 b	10.4 ± 1.3
RTB + WB	49.2 ± 3.2 d	14.1 ± 2.5	47.4 ± 6.9 c	13.6 ± 1.0
SCG	32.9 ± 4.1 c	16.5 ± 0.4	23.6 ± 3.3 b	11.8 ± 0,6
SCG + WB	41.3 ± 3.0 d	21.9 ± 1.9	27.6 ± 3.8 b	14.6 ± 1.8
BS	20.0 ± 1.6 b	3.6 ± 0.1	13.8 ± 2.5 a	2.5 ± 0.8
BS + WB	18.6 ± 1.1 b	3.7 ± 0.2	15.6 ± 3.2 ab	3.1 ± 1.0
DSF	2.7 ± 0.6 a	0.7 ± 0.1	n. d. ‡	n. d. ‡
DSF + WB	3.6 ± 0.8 a	1.0 ± 0.2	n. d. ‡	n. d. ‡

‡ n.d., not detected.

4. Conclusions

The integrated use of different procedures made it possible to acquire information regarding the lignocellulolytic attitudes of *Aspergillus olivimuriae*, a recently described species. The choice of using production waste derived from four different supply chains increased the applicative value of this study. For new taxa, providing information of an applicative nature can be helpful to those who want to undertake screening procedures to evaluate changes to specific materials or productions. Among the tested substrates, RTB was the most conducive to endo-β-1,4-glucanase and cellobiohydrolase productions, due to its high cellulose content, while β-mannanase production was highest in SCG, the hemicellulose of which are mainly composed of glucomannans and galactomannans. In general, the hemicellulolytic attitude of *A. olivimuriae* prevailed over the cellulolytic one, and this behavior was particularly evident in the DSF, where hemicellulose degradation was equal to 60%. Irrespective of the waste used, and as opposed to other *Aspergillus* species, the strain under study did not show ligninolytic potential.

Supplementary Materials: The following Supplementary Materials are available online at https://www.mdpi.com/article/10.3390/app11125349/s1: Excel file, S1_dbCAN2_output_file_A_olivimuriae.xls; Excel file, S2_Annotations.xls.

Author Contributions: Conceptualization, S.C. and A.D.; Formal analysis, E.C. and S.C.; Funding acquisition, M.P.; Investigation, E.C., S.C., C.R. and A.D.; Methodology, E.C.; Supervision, A.D.; Writing—original draft, A.D.; Writing—review & editing, S.C. and M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data is contained within the article or Supplementary Materials.

Acknowledgments: This work was partially supported by the "Departments of Excellence-2018" Program (Dipartimenti di Eccellenza) of the Italian Ministry of Education, University and Research (MIUR), DIBAF-Department of University of Tuscia, Project "Landscape 4.0—food, wellbeing, and environment".

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

Symbols and Abbreviations

BS, barley straw; CAZy, Carbohydrate-Active EnZyme database; DSF, digestate solid fraction; IC, incubation controls; IU, International Units; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MEA, malt extract agar; OM, organic matter; PG, polygalacturonase; PMG, polymethylgalacturonase; SCG, spent coffee grounds; WB, wheat bran; w_{HC}, mass fraction of holocellulose; w_M, mass fraction of moisture in solid-state cultures; w_{WB}, mass fraction of wheat bran in amended cultures; SSC, solid-state cultures; $Y_{GLC/S}$, biomass yield per unit mass of OM consumed.

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