

## Article

# Screening of Fungal Strains for Cellulolytic and Xylanolytic Activities Production and Evaluation of Brewers' Spent Grain as Substrate for Enzyme Production by Selected Fungi

Rossana Liguori <sup>1</sup>, Anna Pennacchio <sup>1</sup>, Luciana Porto de Souza Vandenberghe <sup>2</sup>, Addolorata De Chiaro <sup>1</sup>, Leila Birolo <sup>1</sup> , Carlos Ricardo Soccol <sup>2</sup> and Vincenza Faraco <sup>1,\*</sup>

<sup>1</sup> Department of Chemical Sciences, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Naples, Italy; rossana.liguori@unina.it (R.L.); anna.pennacchio@unina.it (A.P.); addolorata.dechiaro@unina.it (A.D.C.); birolo@unina.it (L.B.)

<sup>2</sup> Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná Coronel Francisco H. Dos Santos Avenue, 210, Curitiba 81531-980, Brazil; lucianapsv@gmail.com (L.P.d.S.V.); soccol@ufpr.br (C.R.S.)

\* Correspondence: vfaraco@unina.it; Tel.: +39-081-674315; Fax: +39-081-674313

**Abstract:** Brewer's spent grain (BSG), the solid residue of beer production, is attracting significant attention as raw material for the production of added value substances, since until recently it was mainly used as animal feed or deposited in landfills, causing serious environmental problems. Therefore, this work aimed at developing a bioprocess using BSG as a substrate for the production of cellulases and xylanases for waste saccharification and bioenergy production. Different fungi were analyzed for their cellulolytic and xylanolytic abilities, through a first screening on solid media by assessment of fungal growth and enzyme production on agar containing carboxymethylcellulose or xylan as the sole carbon source, respectively. The best cellulase and xylanase producers were subjected to quantitative evaluation of enzyme production in liquid cultures. *Aspergillus niger* LPB-334 was selected for its ability to produce cellulase and xylanase at high levels and it was cultivated on BSG by solid state fermentation. The cellulase production reached a maximum of  $118.04 \pm 8.4$  U/g of dry substrate after 10 days of fermentation, while a maximum xylanase production of  $1315.15 \pm 37.5$  U/g of dry substrate was reached after 4 days. Preliminary characterization of cellulase and xylanase activities and identification of the enzymes responsible were carried out.

**Keywords:** fungi; cellulase; xylanase; brewer's spent grain



**Citation:** Liguori, R.; Pennacchio, A.; Vandenberghe, L.P.d.S.; De Chiaro, A.; Birolo, L.; Soccol, C.R.; Faraco, V. Screening of Fungal Strains for Cellulolytic and Xylanolytic Activities Production and Evaluation of Brewers' Spent Grain as Substrate for Enzyme Production by Selected Fungi. *Energies* **2021**, *14*, 4443. <https://doi.org/10.3390/en14154443>

Academic Editor: Devinder Mahajan

Received: 19 March 2021

Accepted: 20 July 2021

Published: 23 July 2021

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2021 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 (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

The actualization of a circular economy through the use of lignocellulosic wastes as renewable resources can lead to reducing the dependence on fossil-based resources and contribute to sustainable waste management, limiting greenhouse gas emissions and environmental impacts [1]. A full recycling and re-use of agro-industrial lignocellulosic wastes take place in second generation biorefinery platforms, where bio-based products (food, feed, chemicals) and bioenergy (biofuels, power and heat) are generated through integrated and sustainable processes [2]. The circular economy, based on the biorefinery platform and the 3Rs approach—reduction, re-use, recycling of waste—aims at accomplishing a closed-loop system to maximize the recovery of raw materials derived from the waste at the end of life. The sustainability and expansion of the circular economy requires a sufficient supply of lignocellulosic biomass, therefore, integrated biorefineries, exploiting the overall lignocellulosic waste components to generate fuels, chemicals and energy, are a pillar of the circular economy [3,4].

Lignocellulosic biomasses—including pulp and paper, agriculture, food, forestry and municipal solid wastes—are considered as the most promising renewable resources, since they are largely available around the world and allow for avoiding the conflict of food

versus fuel linked to the use of edible crops [5]. Lignocellulose's structure consists of a cellulose backbone coated with hemicellulose and lignin in an amorphous three-dimensional structure, with different relative compositions depending on the species. The presence of the recalcitrant lignin requires an efficient treatment to make the (hemi)cellulose more accessible for the subsequent enzymatic hydrolysis, which in turn needs a precisely designed enzymatic cocktail, based on cellulases, hemicellulases and accessory enzymes, due to the complex carbohydrate structure of lignocellulosic biomass [6]. Several methods, including physical, chemical and physicochemical treatments, have been explored to remove lignin, and biological treatment by ligninolytic fungi/enzymes has been applied recently in the production of second-generation biofuels, which are gaining increasing attention due to their environmental advantages [7,8]. Similarly, the enzymatic hydrolysis of the (hemi)cellulose after lignin removal presents environmental advantages in comparison to the chemical method, but poses economical drawbacks due to the high production costs of the involved enzymes [9]. This leads to a search for alternative low-cost substrates based on wastes for enzyme production.

Among the lignocellulosic wastes, brewer's spent grain (BSG) is attracting significant attention. BSG is a solid residue of beer production formed by exhausted grain husks, obtained after mashing and lautering [10]. Until recently, BSG was mainly used as animal feed or deposited in landfills, causing serious environmental problems. Nevertheless, recent studies demonstrated its potential as a substrate to produce several high added value bioproducts, such as organic acids, biogas, bioethanol, biopolymers and molecules for the food and pharmaceutical industries [10–13]. The viability of these processes should be locally evaluated on the basis of BSG availability, its current utilization for animal feed production and the environmental and economical advantages that could be gained by changing its use to generate higher added value products. In our previous work [13], the composition of BSG from the microbrewery Maneba (Striano, Naples, Italy) was determined, revealing very large amounts of cellulose and hemicellulose, and very low lignin content, in comparison to the composition of other types of BSG reported in the literature.

Since cellulases and xylanases are high added value products that can be obtained by waste upgrading through fungal growth [2,3], the aim of this study was to valorize BSG by using it as a substrate for the production of cellulolytic and xylanolytic enzymes by SSF of the fungus *Aspergillus niger* LPB-334, which was selected as the best producer of cellulases and xylanases among 32 fungal strains analyzed for their xylanolytic and cellulolytic abilities.

## 2. Material and Methods

Screening of 32 fungi for cellulase and xylanase production was performed first in solid medium, and the most productive fungi were then cultivated in liquid culture and samples of broth were assayed for cellulase and xylanase activity. The fungus producing both higher levels of cellulase and xylanase activities was selected for the next experiment of solid state fermentation on BSG and cellulase and xylanase activities produced at maximum time of production were subjected to enzyme characterization and protein identification.

### 2.1. Screening of Fungal Strains for Cellulase and Xylanase Production on Solid Medium

Thirty-two fungal strains (*Aspergillus* spp.), listed in Table 1, were grown on potato dextrose yeast (PDY) agar medium for 5 days at 30 °C. The qualitative production of cellulases and xylanases was evaluated through the analysis of the diameter of the formed halo on PDY agar plates containing 1% carboxymethylcellulose (CMC) and 1% xylan, respectively. After incubation for 6 days, the strains were assayed for their ability to degrade CMC/xylan by incubation with 0.1% Congo Red solution for 30 min, followed by washing with 5 M NaCl as reported by Amore et al. [14]. The selection of strains that can efficiently degrade polysaccharides was carried out according to Florencio et al. [15].

**Table 1.** Detection of cellulolytic (A) and xylanolytic (B) enzyme activities on solid medium by 32 fungal strains belonging to DEBB Collection.

Fungal Strains	(A) EI For Cellulase Activity	(B) EI For Xylanase Activity
<i>Lentinus edodes</i> LPB 373 ▲ *	1.6	1.1
<i>Lentinus edodes</i> LPB 374	1.3	1.3
<i>Lentinus edodes</i> LPB 375	1.2	1.2
<i>Lentinus edodes</i> LPB 376 ▲ *	1.4	ND
<i>Lentinus edodes</i> LPB 377 ▲ *	2.2	1.2
<i>Lentinus edodes</i> LPB 378	ND	ND
<i>Lentinus edodes</i> LPB 379	0.9	ND
<i>Lentinus edodes</i> LPB 380	ND	ND
<i>Lentinus edodes</i> LPB 381	1.3	1.25
<i>Lentinus edodes</i> LPB 382	ND	ND
<i>Lentinus edodes</i> LPB 383	ND	ND
<i>Lentinus edodes</i> LPB 384 ▲ ■ *	1.5	1.5
<i>Lentinus edodes</i> LPB 385	1.2	ND
<i>Lentinus edodes</i> LPB 386▲ ■ *	1.4	1.4
<i>Lentinus edodes</i> LPB 387▲ ■ *	1.4	1.4
<i>Pleurotus sajor-caju</i> INRA 31	ND	ND
<i>Pleurotus sajor-caju</i> INRA 3824	1.2	1.3
<i>Trametes versicolor</i> CC124	1.1	1.1
<i>Aspergillus niger</i> LPB-328 ▲ ■ *	1.4	2.0
<i>Aspergillus niger</i> LPB-329 ▲ ■ *	1.5	2.7
<i>Aspergillus niger</i> LPB-330 ▲ ■ *	1.4	1.4
<i>Aspergillus niger</i> LPB-331	ND	ND
<i>Aspergillus niger</i> LPB-334 ▲ ■ *	1.4	1.8
<i>Aspergillus niger</i> LPB-335	1.0	ND
<i>Aspergillus niger</i> LPB-336 ▲ ■ *	1.4	2.0
<i>Aspergillus niger</i> LPB-340 ▲ ■ *	1.5	1.4
<i>Aspergillus niger</i> LPB-349	1.0	1.0
<i>Aspergillus niger</i> LPB-350	1.0	1.3
<i>Aspergillus oryzae</i> LPB-351	ND	ND
<i>Aspergillus oryzae</i> LPB-352	1.2	1.1
<i>Aspergillus oryzae</i> LPB-353	1.1	1.0
<i>Aspergillus oryzae</i> LPB-354	ND	1.1

All values are average of two replicates. The standard deviation is less than 0.1. ND: Not detected. \* Selected fungal strains for quantitative estimation of cellulolytic (▲) and xylanolytic (■) enzyme activities in liquid culture.

## 2.2. Screening for Cellulase and Xylanase Production in Liquid Culture

The quantitative estimation of enzymatic activities employed two different media: medium A (composition ( $\text{g L}^{-1}$ ): yeast extract (0.5);  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.3);  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.005);  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (0.00156);  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.0014);  $\text{CaCl}_2$  (0.3);  $\text{CoCl}_2$  (0.002) and  $\text{KH}_2\text{PO}_4$  (1.5) at pH 5.5, supplemented with cellulose microcrystalline (10.0)) for cellulase and xylanase production analysis [14], and medium B [15]. Three agar cores from *Lentinus* spp., *Pleurotus* spp. and *Trametes* spp. (1.3 mm in diameter) from 6-day-old cultures were pre-inoculated into 50 mL of PDY medium and, after 6 days, 10% of the inoculum was transferred to medium A or B for enzyme production. Regarding *Aspergillus* spp., the spore suspension was used to inoculate medium A or B at a final concentration of  $10^7$  spores  $\text{g}^{-1}$  of the carbon source. Flasks were incubated at 30 °C, 120 rpm and sampling for enzymatic quantification was performed daily for 25 days.

### 2.3. Determination of Protein Concentration

Protein concentration of a crude enzyme preparation was evaluated using the Bradford reagent of BioRad (München, Germany) following the supplier's instructions and using bovine serum albumin (BSA) as the standard protein.

### 2.4. Enzyme Production Using Brewer's Spent Grain

Spore suspension and pre-inoculum production of *Aspergillus* spp. were performed as reported in Montibeller Weingartner et al. [16]. Approximately  $10^7$  spores  $g^{-1}$  were inoculated on 25 g of dry BSG, in 250 mL Erlenmeyer flasks, in duplicate. The initial moisture was adjusted to 70% by adding enough volume of distilled water and a mineral salt solution containing (g/L):  $KH_2PO_4$  (1.5),  $CuSO_4$  (0.4) and  $CoSO_4$  (0.0012). The flasks were incubated statically at 30 °C for 16 days. Enzyme extraction was performed according to Montibeller Weingartner et al. [16].

### 2.5. Enzyme Assays

CMCase and xylanase activity assays were carried out according to Ghose [17] and Bailey et al. [18] Released reducing sugars were determined by the dinitros-lyclic acid reagent (DNS) method [19].

### 2.6. Preliminary Characterization of Cellulase and Xylanase from *Aspergillus niger* LPB-334

Enzymes produced by *Aspergillus niger* LPB-334 SSF were extracted by solid-liquid extraction using 50 mM sodium citrate buffer, pH 5.0, in a proportion of 1:10 (*w/v*) and incubated at room temperature, 120 rpm for 10 min. The mix was filtered with TNT paper and centrifuged at 8000 rpm, 4 °C for 15 min. The supernatants containing cellulase and xylanase were filtered using Whatman n° 1 filter paper and concentrated by using the stirred ultrafiltration Amicon®system (Millipore Corporation, Bedford, MA, USA) with a 10 kDa polyethersulfone membrane. The concentrated enzymatic extract was used to determine the optimum temperature, pH, thermoresistance and pH resistance of the cellulase and xylanase activities. The optimum temperature of cellulase activity was assessed at 37, 45, 50, 55 and 60 °C by using Azo-CMC (Megazyme, Ireland) dissolved in 50 mM potassium citrate at pH 5.0 as a substrate, following the supplier's instructions [14]. The thermoresistance was evaluated at 37, 45, 50, 55 and 60 °C for 72 h in 50 mmol L<sup>-1</sup> Na citrate at pH 7.0. In order to determine the optimum pH and pH resistance of cellulase activity, enzymatic essays were carried out at 50 °C at pH values between 3.0 and 7.0, using Azo-CMC (Megazyme, Co. Wicklow Ireland) as a substrate dissolved in 50 mM potassium citrate buffer for pH 3–5 and in 50 mM potassium phosphate buffer for pH 6.0 and pH 7.0. To determine the optimum temperature of xylanase activity, the substrate of the activity assay (birch-wood xylan) was dissolved in 50 mmol L<sup>-1</sup> Na citrate at pH 5.0 and the incubation (10 min) was performed at 37, 45, 50, 55 and 60 °C. The thermostability of xylanase activity was estimated at 37, 45, 50, 55 and 60 °C for 72 h in 50 mmol L<sup>-1</sup> Na citrate at pH 7.0. In order to determine the optimum pH and pH resistance of xylanase activity, the substrate birch-wood xylan was dissolved in 50 mM potassium citrate buffer for pH 3–5 and in 50 mM potassium phosphate buffer for pH 6.0 and pH 7.0 and incubated for 10 min at 50 °C. Samples were withdrawn for a residual xylanase activity assay as described above.

### 2.7. Zymogram Analyses and Protein Identification by Mass Spectrometry

Semi-denaturing gel electrophoresis was performed by loading nondenatured and not-reduced samples on a SDS polyacrylamide gel, as described by Laemmli [20]. Proteins showing cellulolytic and xylanolytic activities were visualized according to Amore et al. [21] and Amore et al. [22], respectively. Slices showing the activities of interest were cut from the semi-denaturing PAGE and analyzed by mass spectrometry as reported in Amore et al. [21]. Briefly, Coomassie-stained semi-denaturing PAGE and zymogram analyses were aligned to select bands of interest, which were cut from the semi-denaturing PAGE, destined by

extensive alternated washes with 0.1 mol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> pH 7.5 and acetonitrile, then reduced for 45 min in 100 µL of 10 mmol L<sup>-1</sup> dithiothreitol, 0.1 mol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 and carboxyamidomethylated for 30 min in the dark by the addition of 100 µL of 55 mM iodoacetamide dissolved in the same buffer. Enzymatic digestion was performed by adding to each slice 100 ng of proteomic-grade trypsin (SIGMA) in 10 µL of 10 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.5 for 2 h at 4 °C. The buffer solution was then removed and 50 µL of 10 mM NH<sub>4</sub>HCO<sub>3</sub> pH 7.5 were added and incubated for 18 h at 37 °C. Peptides were extracted with 20 µL of 10 mM NH<sub>4</sub>HCO<sub>3</sub>, 1% formic acid, 50% acetonitrile at room temperature.

Peptide mixtures were filtered on 0.22 µm PVDF membranes (Millipore) and spin filter membranes (Agilent Technologies) and analyzed by LC-MS/MS on a 6520 Accurate-Mass Q-TOF LC-MS System (Agilent Technologies, Palo Alto, CA, USA) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed in a 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at a flow rate of 400 nL min<sup>-1</sup>, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% in 50 min.

Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 *m/z*) followed by MS/MS scans of the five most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50,000 counts. Double- and triple-charged ions were preferably isolated and fragmented. The acquired MS/MS spectra were transformed into Mascot generic format (.mgf) and used for protein identification in the unreviewed set of protein entries that are present in the NCBIInr database for all fungi (NCBIInr 20150220 (61,078,976 sequences; 21,793,310,741 residues) or in the Uniprot database for all fungi (SwissProt 2015\_02 (547,599 sequences; 195,014,757 residues), with a licensed version of Mascot software ([www.matrixscience.com](http://www.matrixscience.com)) version 2.4.0. Additional Mascot search parameters were: peptide mass tolerance 10 ppm, fragment mass tolerance 0.6 Da, up to 3 missed cleavages, carbamidomethylation of cysteines as fixed modification, oxidation of methionine, pyro-Glu N-term Q, as variable modifications. Only doubly and triply charge ions were considered. Ion score was  $-10 \log(P)$ , where P is the probability that the observed match is a random event. Individual ion scores > 45 indicated identity or extensive homology ( $p < 0.05$ ). Protein scores were derived from ion scores as a nonprobabilistic basis for ranking protein hits ([http://www.matrixscience.com/help/interpretation\\_help.html](http://www.matrixscience.com/help/interpretation_help.html)).

### 2.8. Statistical Analyses

One-way ANOVA followed by Duncan's HSD post hoc test for pairwise comparison of means (at  $p < 0.05$ ) was used to assess the difference in the xylanase and cellulase activities of microbial strains using the SPSS 21.0 software package (SPSS Inc., Cary, NC, USA).

## 3. Results and Discussion

### 3.1. Evaluation of Enzyme Production on Solid Medium

Thirty-two fungal strains from the DEBB Collection, belonging to the genera *Lentinus*, *Pleurotus*, *Trametes* and *Aspergillus* spp., were analyzed for their cellulolytic and xylanolytic abilities by assessment of fungal growth and enzyme production on agar plates containing carboxymethylcellulose (CMC) or xylan as the sole carbon source, respectively (Table 1). All strains were able to grow on these substrates, showing their capacity to produce enzymes that can metabolize them. The screening was performed by a Congo Red test, based on the formation of a halo around the fungal colony, corresponding to the region of action of (hemi)cellulolytic enzymes. In fact, the Congo Red dye only remains attached to regions where  $\beta$ -1,4-D-glucanohydrolase bonds are not hydrolyzed [23,24]. The selection of strains that can efficiently degrade polysaccharides was carried out by evaluating the enzymatic index (EI), the ratio between the diameter of the hydrolysis halo and the diameter of the fungal mycelium. Fungal strains that showed an EI equal or higher than 1.4 were selected

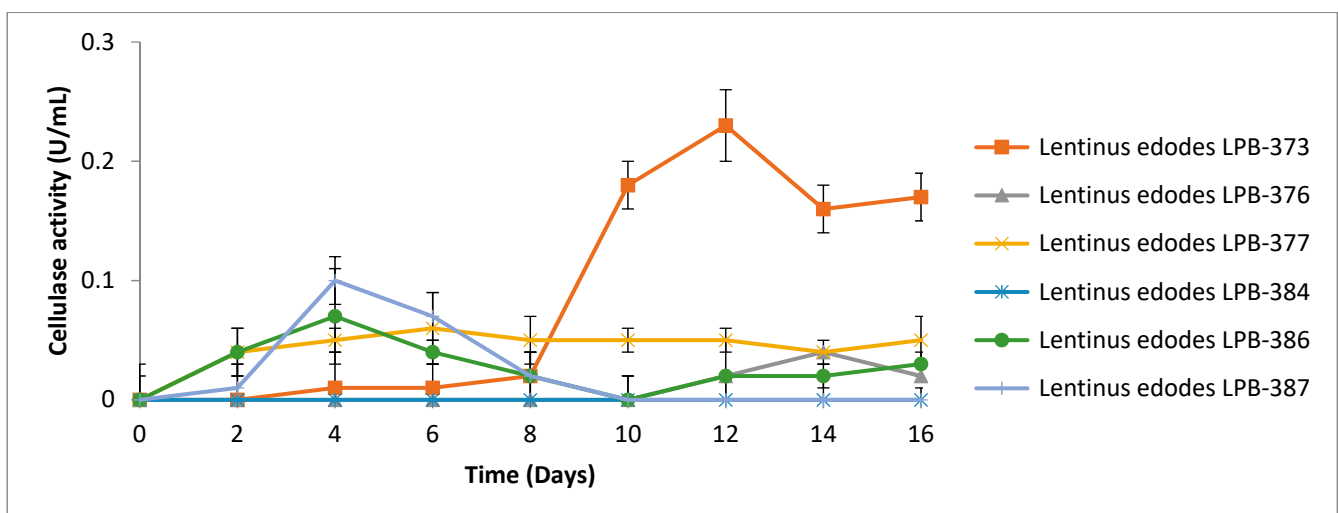
for the subsequent investigation of cellulase and xylanase production in liquid culture. The EI measured for the investigated fungal strains after 5 days of incubation at 30 °C is shown in Table 1. The reported values are the average of measurements for two independent experiments carried out in the same conditions.

*L. edodes* LPB 373 (EI = 1.6), LPB 374 (EI = 1.3) and LPB 377 (EI = 2.2) were selected for cellulase activity production, whilst the strains *Lentinus edodes* LPB 384 (EI cell = 1.5 and EI xyl = 1.5), LPB 386 (EI cell = 1.4 and EI xyl = 1.4) and LPB 387 (EI cell = 1.4 and EI xyl = 1.4) and the strains *A. niger* LPB-328 (EI cell 1.4 and EI xyl = 2.0), LPB-329 (EI cell = 1.5 and EI xyl = 2.7), LPB-330 (EI cell = 1.4 and EI xyl = 1.4), LPB-334 (EI cell = 1.4 and EI xyl = 1.8), LPB-336 (EI cell = 1.4 and EI xyl = 2.0) and LPB-340 (EI cell = 1.5 and EI xyl = 1.4) were selected for the evaluation of cellulase and xylanase activities. All these strains were, therefore, further analyzed for a quantitative estimation of cellulase and xylanase production in liquid culture.

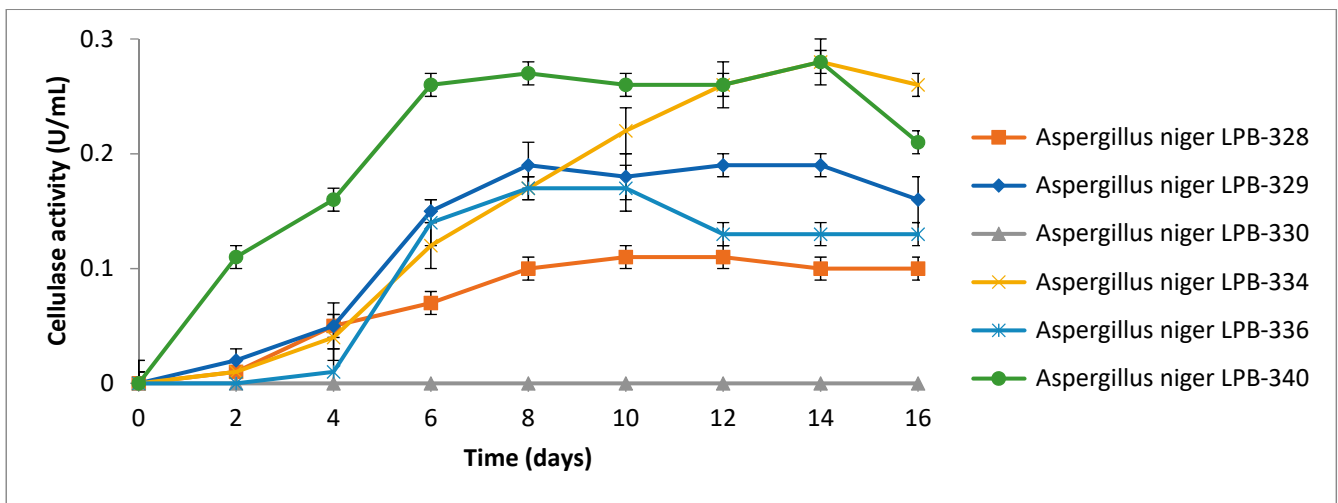
### 3.2. Evaluation of Enzyme Production in Liquid Medium

The 12 fungal strains selected for cellulase production on solid medium, taking the enzymatic index value into account, were subjected to a quantitative evaluation of cellulase production in liquid culture containing microcrystalline cellulose as the carbon source. The screening showed that the strain *L. edodes* LPB 373 reached a maximum of cellulase activity of  $0.23 \pm 0.05$  U/mL ( $p < 0.05$ ) after 12 days of fermentation (Figure 1) and the strains *A. niger* LPB-334 and *A. niger* LPB-340 achieved  $0.27 \pm 0.04$  U/mL ( $p < 0.05$ ) and  $0.28 \pm 0.04$  U/mL ( $p < 0.05$ ) of cellulase activity, respectively, after 14 days of fermentation. Moreover, it is worth noting that the latter strain reached  $0.27 \pm 0.04$  U/mL ( $p < 0.05$ ) of cellulases after 6 days of fermentation (Figure 2), which means a higher productivity ( $0.043$  U/mL day) than *A. niger* LPB 334 ( $0.022$  U/mL day).

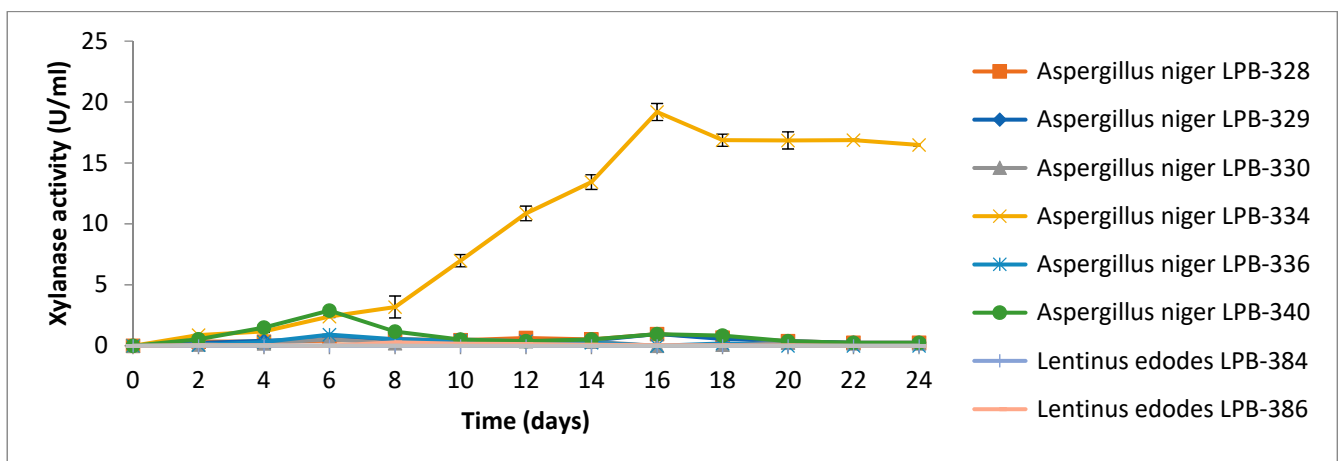
For the nine strains previously selected as xylanase producers in solid medium, the time course of xylanase production was followed in liquid culture containing microcrystalline cellulose as the carbon source. *A. niger* LPB-334, showing the highest xylanase activity of  $19.18 \pm 6.2$  U/mL after 16 days (Figure 3;  $p < 0.05$ ), was demonstrated to be by far the best xylanase activity producer. Further incubation did not show any increment in the level of enzyme production, probably due to depletion of nutrients in the media.



**Figure 1.** Time course of cellulase activity production in liquid culture by *Lentinus edodes* strains. The error bars represent standard deviations. The averages of results of three independent experiments, each one performed with three biological replicates, are reported.



**Figure 2.** Time course of cellulase activity production in liquid culture by *Aspergillus niger* strains. The error bars represent standard deviations. The averages of results of three independent experiments, each one performed with three biological replicates, are reported.

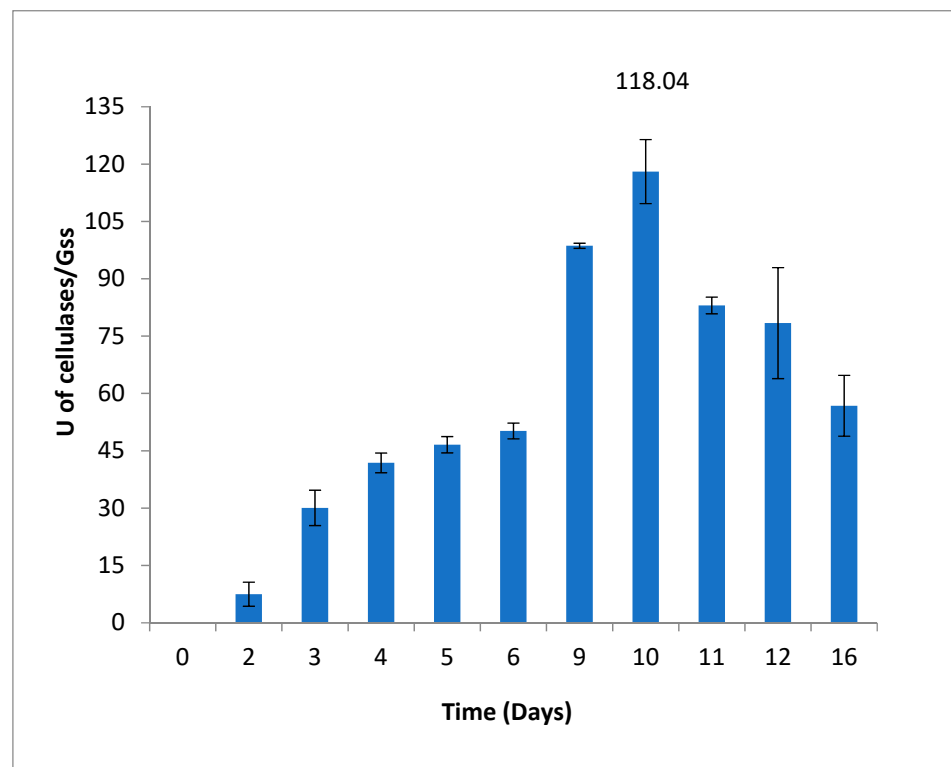


**Figure 3.** Time course of xylanase activity production in liquid culture by *Aspergillus niger* and *Lentinus edodes* strains. The error bars represent standard deviations. The averages of results of three independent experiments, each one performed with three biological replicates, are reported.

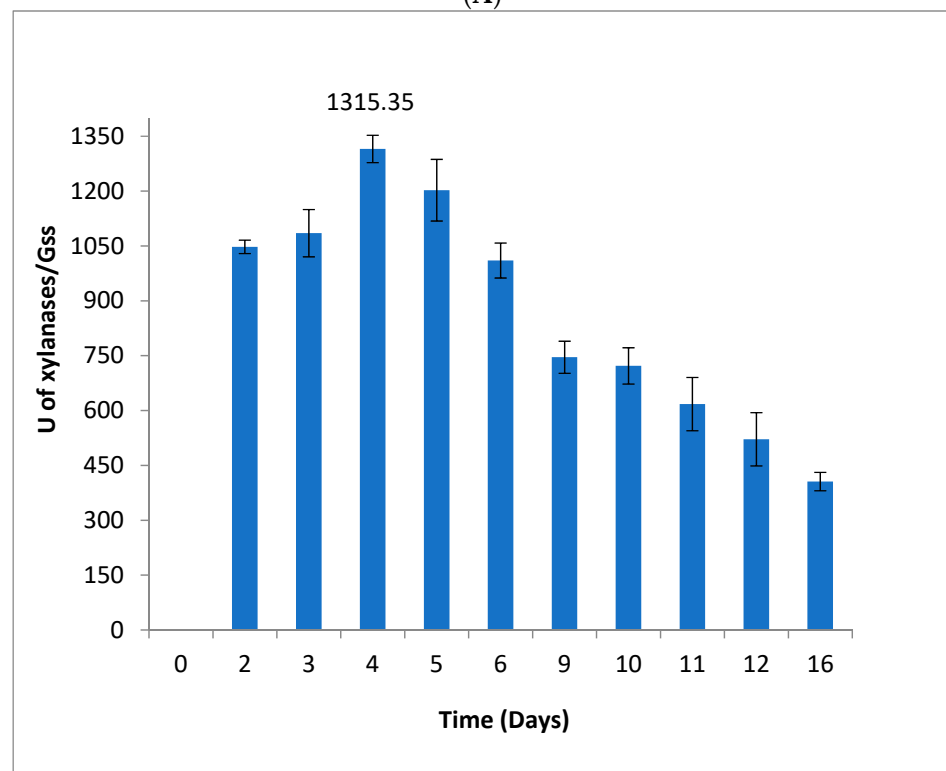
The strain *Aspergillus niger* LPB-334 was selected for further experiments since it was demonstrated to be the best producer of both cellulase and xylanase activities in liquid culture.

### 3.3. Evaluation of Enzyme Production by SSF Using BSG

The strain *Aspergillus niger* LPB-334, selected for further experiments since it was demonstrated to be the best producer of both cellulase and xylanase activities in liquid culture, was tested for its ability to grow on BSG solid medium and to produce cellulases and xylanases. The time course of cellulase activities revealed a maximum activity of  $118.04 \pm 8.4$  U/g of dry substrate after 10 days of fermentation (Figure 4A), while, as for the xylanase production, a maximum of  $1315.15 \pm 37.5$  U/g of dry substrate was reached after 4 days of fermentation (Figure 4B).



(A)



(B)

**Figure 4.** Time course of (A) cellulase and (B) xylanase activities produced by *Aspergillus niger* LPB-334 in SSF on BSG. The error bars represent standard deviations. The averages of results of three independent experiments, each one performed with three biological replicates, are reported.



A higher value of xylanase production compared to cellulase production is observed in most of *Aspergillus* spp. [25–29]. When compared with the xylanase production by SSF reported for other *Aspergillus* spp., the maximum value of U/mL achieved by *A. niger* LPB-334 was higher than some fungi [16,29–34] and lower than others [25,35,36] (Table 2).

**Table 2.** Comparison of cellulase and xylanase production by other *Aspergillus* spp.

Microorganism	Substrate	Maximum Xylanase Activity	Maximum Cellulase Activity	References
<i>A. niger</i> M85	Rice straw	1493.1 U/g	13.67 U/g	[35]
<i>Aspergillus terreus</i>	Rumen and feces by culture in cellulose-based medium.	54.84 U/mg	33.03 U/mg	[34]
<i>A. niger</i> KK2	Rice straw	5070 U/gds	19.5 U/gds FPase 129 U/gds CMCCase, 100 U/gds $\beta$ -glucosidase	[25]
<i>A. niger</i> USM AI 1	Sugarcane bagasse and palm kernel cake	-	3.4 U/gds FPU	[37]
<i>A. niger</i> From Dharmapuri Dist, Tamil Nadu, India	Coir waste	-	8.89 U/gds CMCCase, 3.56 U/gds FPase	[38]
<i>A. niger</i> JL-15	Orange peel	917.7 U/gds	-	[30]
<i>Aspergillus niger</i> NCIM 563	Wheat bran	133.2 IU/g	41.58 IU/g	[29]
<i>A. niger</i> F-3	Citrus peels	250 U/gds	-	[31]
<i>A. niger</i> NS-2	Wheat bran	-	17 U/gds CMCCase, 310 U/gds FPase 33 U/gds $\beta$ -glucosidase	[39]
<i>A. niger</i> FGSCA733	<i>Jatropha curcas</i> seed cake	6087 U/gds	3974 U/gds	[26]
<i>A. fumigatus</i> SK1	Untreated oil palm trunk	418.7 U/gds	54.27 U/gds CMCCase, 3.36 U/gds FPase 4.54 U/gds $\beta$ -glucosidase	[27]
<i>A. fumigatus</i> P40M2 and <i>A. niger</i> P47C3	Wheat bran	1055.6 U/gds 1258.0 U/gds	105.8 U/gds $\beta$ -glucosidase 96.0 U/gds $\beta$ -glucosidase	[28]
<i>Aspergillus niger</i> HN-1	Rice straw of variety PR-127	2593.5 IU/gds xylanase,	36.7 FPU/gds FPase, 252.3 IU/gds $\beta$ -glucosidase, 416.3 IU/gds endoglucanase	[36]
<i>A. niger</i> from Rio de Janeiro, Brazil	Sugar bagasse	26.1 U/g	0.4 U/g FPase, 14.9 U/g CMCCase	[32]
<i>A. niger</i> NRRL 328	Soybean husks	950 U/gds	-	[16]
<i>Aspergillus niger</i> (strain—113 N) and <i>Aspergillus fumigatus</i> (strain—3)	Grape skin Olive pomace	47.05 U/g 8 U/g	10 U/g CMCCase, 29.47 U/g $\beta$ -glucosidase 6 U/g CMCCase, 10.02 U/g $\beta$ -glucosidase	[33]

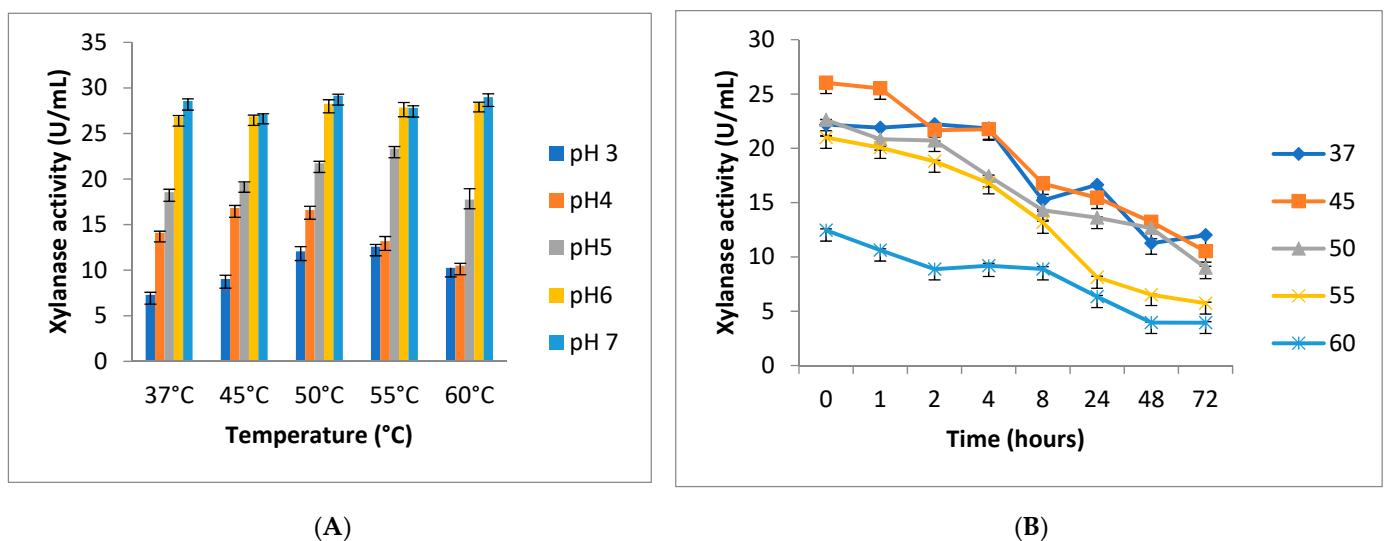
As far as cellulase production is concerned, the maximum value achieved by *A. niger* LPB 334 was, in most cases, higher [29,32,33,37–39] and only in a few cases lower [26,36] than reported for other *Aspergillus* strains (Table 2).

Taking into consideration that no optimization was carried out in our study and no additional nutrient was adopted, this process presents high expectations for abundant cellulase and xylanase production.

### 3.4. Preliminary Enzyme Characterization

Optimal temperature, pH, thermoresistance and pH resistance of cellulase and xylanase of *Aspergillus niger* LPB-334 were evaluated in the enzymatic extract produced by SSF on BSG on the 10th day (maximum of production).

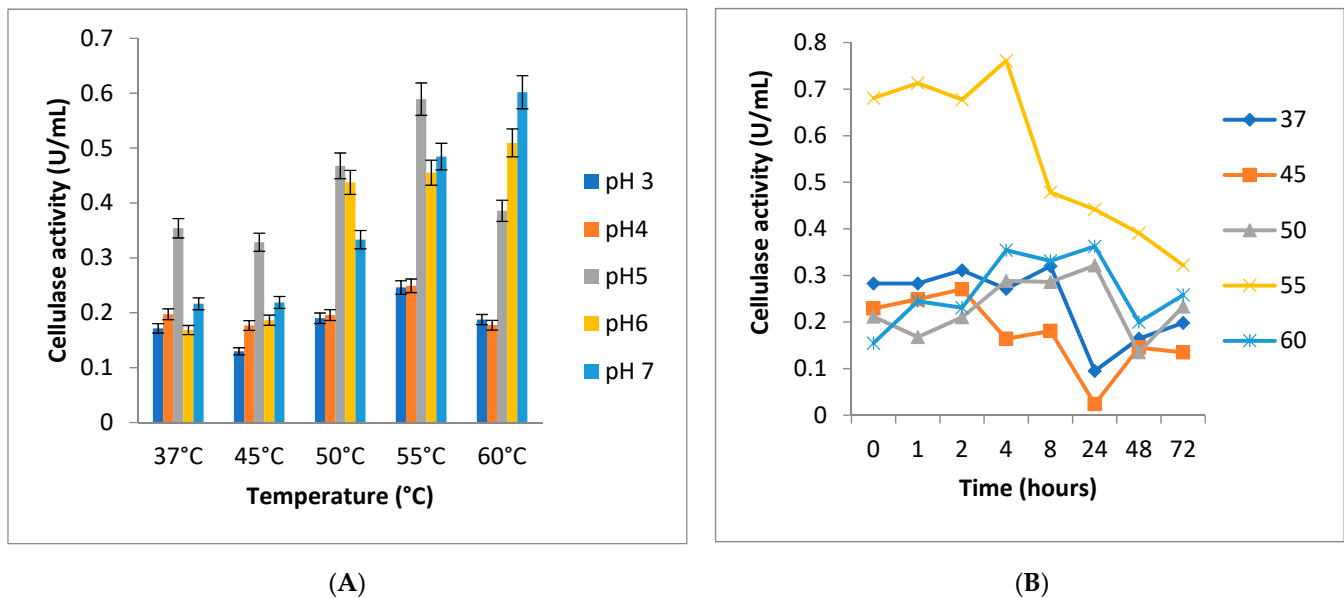
The xylanase activity shows an optimum at pH of 7 and temperature of 50 °C ( $28 \text{ U/mL} \pm 0.12$ ), as reported in Figure 5A, although at neutral pH, no substantial differences can be observed in the temperature range 37–60 °C. Conversely, at pH below 6, it visibly reduces its activity at all the temperatures tested (Figure 5A). The optimal temperature is almost equal to that of xylanase from *Aspergillus awamori* [40], and higher than the optimal temperature (30 °C) exhibited by the xylanase from *Aspergillus fumigatus* [41] and xylanase from *Aspergillus niger* [42]. The optimal pH is similar to that of the xylanase from *Aspergillus fumigatus* [41] and xylanase from *Aspergillus niger* [42] while xylanase from *Aspergillus awamori* exhibited an optimal pH of 5 [40]. Thermoresistance of xylanase is shown in Figure 5B. The enzymatic activity is quite stable even for prolonged incubation if the extract is preserved in a range of pH from 4 to 7 and a temperature between 37 °C and 60 °C (up to 8 h). At the temperature of BSG bioconversion (37 °C), the enzyme retained 100% activity even after 4h of incubation. When compared to the other fungal xylanases characterized, our enzyme shows similar or better thermoresistance. As a matter of fact, xylanase from *Aspergillus fumigatus* [41] retained 100% activity at 30 °C after 4 h of incubation, xylanase from *Aspergillus awamori* [40] retained 60% activity at 30 °C after 1 h of incubation and xylanase from *Aspergillus niger* retained 50% activity at 30 °C after 30 min of incubation. It is worth noting that after 24 h, the enzyme retains 72% activity and, even after 72 h, the enzyme still has 59% residual activity.



**Figure 5.** (A) Effect of temperature and pH on xylanase activity from *Aspergillus niger* LPB-334. (B) Thermoresistance of xylanase activity from *Aspergillus niger* LPB-334. The error bars represent standard deviations. The averages of results of three independent experiments, each one performed with three biological replicates, are reported.

As far as the enzymatic activity of cellulase is concerned, activity is almost constant in the range between pH 5 and pH 7 but the activity decreases at pH values below 5 (Figure 6A). The cellulase activity shows an optimum pH of 7 and an optimum temperature of 60 °C ( $0.4 \text{ U/mL} \pm 0.05$ ). The optimal temperature is higher than the optimal temperature (55 °C) exhibited by the cellulase from *Aspergillus niger* VTCC-F021 [43] and the optimal temperature (50 °C) of cellulase from *Bacillus amyloliquefaciens* [44]. The optimal pH is higher than the optimal pH (5) of the cellulase from *Aspergillus niger* VTCC-F021 [43] but lower than the optimal pH (8) of cellulase from *Bacillus amyloliquefaciens* [44]. Figure 6B reports data on cellulase thermoresistance. The enzymatic activity is quite stable when the extract is kept at a pH in the range from 4 to 7 and at temperatures between 37 °C and

60 °C. At 37 °C, the enzyme retains 100% activity up to 2 h of incubation, 87% activity was retained after 8 h and, after 24 h, the protein still had 50% residual activity. In comparison to the other fungal cellulases characterized, our enzyme shows similar or even better thermoresistance, as can be inferred by the observation that the cellulase from *Aspergillus niger* VTCC-F021 [43] after 8 h incubation at 37 °C retains 60% activity while cellulase from *Bacillus amyloliquefaciens* [44] has 60% residual activity at 40 °C after 20 h of incubation.



**Figure 6.** (A) Effect of temperature and pH on cellulase activity produced by *Aspergillus niger* LPB-334. (B) Thermoresistance of cellulase activity from *Aspergillus niger* LPB-334. The error bars represent standard deviations. The averages of results of three independent experiments, each one performed with three biological replicates, are reported.

### 3.5. Identification of Novel Cellulases and Xylanases from *A. niger* LPB-334

The identification of novel cellulases and xylanases can be very important in the perspective of their potential exploitation in industrial processes for the conversion of lignocellulosic biomasses into second-generation biofuels or other high added value bio-products. Due to the presence of lignin in cellulosic substrates and the crystalline nature of cellulose, it is inaccessible for enzyme action, unless there is a synergistic action between these enzymes. Cellulase breaks cellulose into glucose sugars and xylanase helps by breaking down xylans to xylose which is also a fermentable sugar. Additionally, the breaking down of xylan by xylanase further improves the cellulose fiber porosity and makes it more accessible to cellulases [45].

Enzymes putatively responsible for the cellulase and xylanase activities in *A. niger* LPB-334 were tentatively identified after a fractionation on a semi-denaturing SDS-PAGE, one for each activity, where samples from the supernatant of the cell cultures were loaded without any denaturing treatment.

Five cellulolytic activity-positive bands were visualized by zymography (Figure 7), as described in the Materials and Methods section. Slices corresponding to these active bands were excised from the Coomassie-stained semi-denaturing SDS-PAGE and subjected to protein identification after in situ digestion and LC-MS/MS analysis of the peptide mixtures, in a classical bottom-up proteomic approach, to identify the putative cellulases and xylanases responsible for the enzymatic activity. Raw data were used to query the nonredundant NCBI or Uniprot databases available on the net with the MS/MS ion search program on a Mascot server, with fungi as the taxonomic restriction, as described in the Materials and Methods section.

**Table 3.** Cellulase identified in the supernatant of *Aspergillus niger* LPB-334 strain. Bands were selected on the basis of zymograms as shown in Figure 7 and excised from the gel, in situ digested with trypsin and the resulting peptide mixtures were analyzed by LC-MS/MS. Band number corresponds to the band selected on the gel. Raw data were used to query the NCBI database with the taxonomic restriction to fungi as described in detail in the Materials and Methods section. Only proteins identified with two or more peptides were considered as confidently identified. Only proteins that have been annotated in the database as being involved in cellulose metabolism have been reported.

Band	GI Number Identifier	UniProtKB ID (When Available)	Entry Name	Protein Name [Strain]	Protein Score <sup>a</sup>	N Peptides	Sequence Coverage (%)
1	145255120	A2RAR6	EXGA_ASPNC	Probable glucan 1,3-beta-glucosidase A [ <i>Aspergillus niger</i> CBS 513.88]	946	18	56%
	74698498	Q9UVS8	CBHB_ASPNG	1,4-beta-D-glucan cellobiohydrolase B	316	6	24%
	145230419	A2QAC9	A2QAC9_ASPNC	Glycosidase [ <i>Aspergillus niger</i> CBS 513.88]	270	6	20%
	317028369			Glucan endo-1,6-glucosidase BGN16.3 [ <i>Aspergillus niger</i> CBS 513.88]	229	4	23%
	298351856	A2QH21	EGLC_ASPNC	Probable glucan endo-1,3-beta-glucosidase egIC [ <i>Aspergillus niger</i> CBS 513.88]	146	3	7%
	145246118	A2QYR9	CBHC_ASPNC	Probable 1,4-beta-D-glucan cellobiohydrolase C [ <i>Aspergillus niger</i> CBS 513.88]	144	4	22%
	145238644	A2QPC3	EGLB_ASPNC	Probable endo-beta-1,4-glucanase B [ <i>Aspergillus niger</i> CBS 513.88]	140	2	10%
	3757552	O74705	O74705_ASPNG	Acetyltransferase (GNAT) family protein [ <i>Aspergillus niger</i> ]	113	2	15%
	145255120	A2RAR6	EXGA_ASPNC	Probable glucan 1,3-beta-glucosidase A [ <i>Aspergillus niger</i> CBS 513.88]	1095	22	68%
2	145256130	A2QUZ1	A2QUZ1_ASPNC	1,3-beta-glucanosyltransferase [ <i>Aspergillus niger</i> CBS 513.88]	788	15	39%
	317028369			Glucan endo-1,6-glucosidase BGN16.3 [ <i>Aspergillus niger</i> CBS 513.88]	448	7	33%
	74698498	Q9UVS8	CBHB_ASPNG	1,4-beta-D-glucan cellobiohydrolase B	409	8	25%
	145238644	A2QPC3	EGLB_ASPNC	Probable endo-beta-1,4-glucanase B [ <i>Aspergillus niger</i> CBS 513.88]	297	4	14%
	145230419	A2QAC9	A2QAC9_ASPNC	Glycosidase [ <i>Aspergillus niger</i> CBS 513.88]	242	5	19%
	145246118	A2QYR9	CBHC_ASPNC	1,4-beta-D-glucan cellobiohydrolase C [ <i>Aspergillus niger</i> CBS 513.88]	222	5	35%
	3757552	O74705	O74705_ASPNG	Acetyltransferase (GNAT) family protein [ <i>Aspergillus niger</i> ]	194	4	20%
	298351856	A2QH21	EGLC_ASPNC	Probable glucan endo-1,3-beta-glucosidase egIC [ <i>Aspergillus niger</i> CBS 513.88]	141	3	7%
	145230537	A2QAI8	A2QAI8_ASPNC	Endo-beta-1,4-mannanase F [ <i>Aspergillus niger</i> CBS 513.88]	138	4	10%

Table 3. Cont.

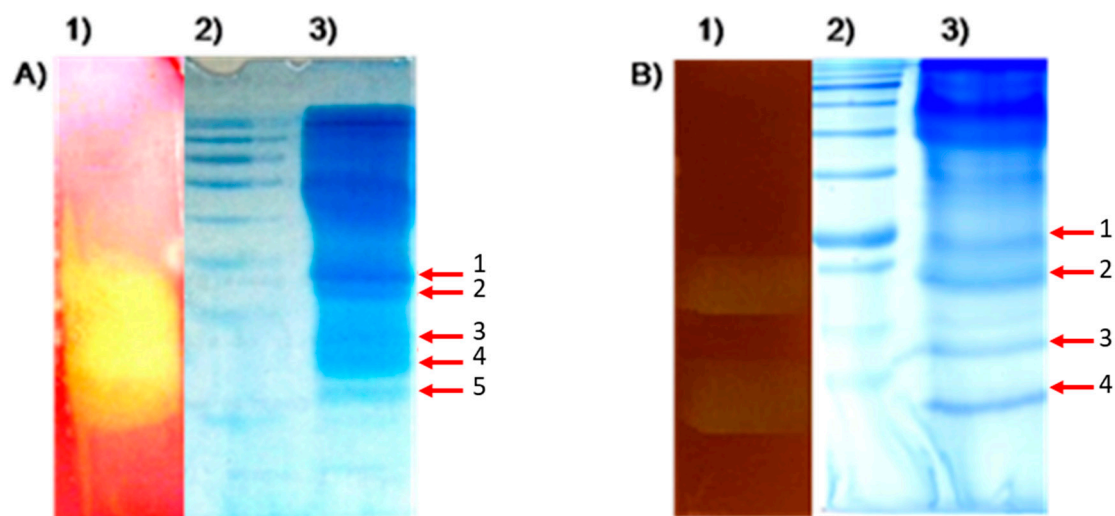
Band	GI Number Identifier	UniProtKB ID (When Available)	Entry Name	Protein Name [Strain]	Protein Score <sup>a</sup>	N Peptides	Sequence Coverage (%)
3	145238644	A2QPC3	EGLB_ASPNC	Probable endo-beta-1,4-glucanase B [ <i>Aspergillus niger</i> CBS 513.88]	698	10	32%
	74698499	Q9UVS9	CBHA_ASPNG	1,4-beta-D-glucan cellobiohydrolase A [ <i>Aspergillus niger</i> ]	673	10	38%
	145255120	A2RAR6	EXGA_ASPNC	Probable glucan 1,3-beta-glucosidase A [ <i>Aspergillus niger</i> CBS 513.88]	552	10	45%
	298351856	A2QH21	EGLC_ASPNC	Probable glucan endo-1,3-beta-glucosidase eglC [ <i>Aspergillus niger</i> CBS 513.88]	462	6	26%
	145246118	A2QYR9	CBHC_ASPNC	Probable 1,4-beta-D-glucan cellobiohydrolase C [ <i>Aspergillus niger</i> CBS 513.88]	219	5	27%
	145230537	A2QAI8	A2QAI8_ASPNC	Endo-beta-1,4-mannanase F [ <i>Aspergillus niger</i> CBS 513.88]	201	4	13%
	145230419	A2QAC9	A2QAC9_ASPNC	Glycosidase [ <i>Aspergillus niger</i> CBS 513.88]	111	4	25%
4	145238644	A2QPC3	EGLB_ASPNC	Probable endo-beta-1,4-glucanase B [ <i>Aspergillus niger</i> CBS 513.88]	828	13	47%
	298351856	A2QH21	EGLC_ASPNC	Probable glucan endo-1,3-beta-glucosidase eglC [ <i>Aspergillus niger</i> CBS 513.88]	606	11	27%
	74698498	Q9UVS8	CBHB_ASPNG	1,4-beta-D-glucan cellobiohydrolase B	599	11	37%
	145230537	A2QAI8	A2QAI8_ASPNC	Endo-beta-1,4-mannanase F [ <i>Aspergillus niger</i> CBS 513.88]	408	5	14%
5	145238644	A2QPC3	EGLB_ASPNC	Probable endo-beta-1,4-glucanase B [ <i>Aspergillus niger</i> CBS 513.88]	516	8	24%
	145230537	A2QAI8	A2QAI8_ASPNC	Endo-beta-1,4-mannanase F [ <i>Aspergillus niger</i> CBS 513.88]	470	6	14%
	298351856	A2QH21	EGLC_ASPNC	Probable glucan endo-1,3-beta-glucosidase eglC [ <i>Aspergillus niger</i> CBS 513.88]	404	6	23%

<sup>a</sup> Protein scores are derived from ion scores as a nonprobabilistic basis for ranking protein hits ([http://www.matrixscience.com/help/interpretation\\_help.html](http://www.matrixscience.com/help/interpretation_help.html)). Ion score is  $-10 \log(P)$ , where  $p$  is the probability that the observed peptide match is a random event.

**Table 4.** Xylanase identified in the supernatant of *Aspergillus niger* LPB-334 strain. Bands were selected on the basis of zymograms as shown in Figure 7 and excised from the gel, in situ digested with trypsin and the resulting peptide mixtures were analyzed by LC-MS/MS. Band number corresponds to the band selected on the gel. Raw data were used to query the NCBI database with the taxonomic restriction to fungi as described in detail in the Materials and Methods section. Only proteins identified with two or more peptides were considered as confidently identified. Only proteins that have been annotated in the database as being involved in the metabolism of xylan have been reported.

Band	GI Number Identifier	UniProtKB ID	Entry Name	Protein Name	Protein Score <sup>a</sup>	N Peptides	Sequence Coverage (%)
1	168481219	P55330	XYNB_ASPNG	Endo-1,4-beta-xylanase B [ <i>Aspergillus niger</i> ]	178	2	14
	239586436	C5J411	XYNC_ASPNG	Probable endo-1,4-beta-xylanase C- [ <i>Aspergillus niger</i> ]	129	3	13
	145230215	A2QA27	XYND_ASPNC	Probable exo-1,4-beta-xylosidase xlnD [ <i>Aspergillus niger</i> CBS 513.88]	108	3	9
2	168481219	P55330	XYNB_ASPNG	Endo-1,4-beta-xylanase B [ <i>Aspergillus niger</i> ]	205	3	15
	145230215	A2QA27	XYND_ASPNC	Probable exo-1,4-beta-xylosidase xlnD [ <i>Aspergillus niger</i> CBS 513.88]	141	4	7
4	168481219	P55330	XYNB_ASPNG	Endo-1,4-beta-xylanase B [ <i>Aspergillus niger</i> ]	105	2	14

<sup>a</sup> Protein scores are derived from ion scores as a nonprobabilistic basis for ranking protein hits ([http://www.matrixscience.com/help/interpretation\\_help.html](http://www.matrixscience.com/help/interpretation_help.html)). Ion score is  $-10 \log(P)$ , where  $p$  is the probability that the observed peptide match is a random event.



**Figure 7.** Protein fractionation on semi-denaturing gel of supernatants of *Aspergillus niger* LPB-334 strain. Semi-denaturing SDS-PAGEs (12.5%) of *Aspergillus niger* LPB-334 extracts without denaturing and reducing treatments: zymograms of cellulase activity (A, lane 1) and xylanase activity (B, lane 1) were aligned to corresponding lanes stained by Coomassie Blue (A,B, lane 3) to cut protein bands as labeled by arrows. Zymogram analysis for cellulase and xylanase detection was carried out on agar plate with 1% CMC and 1% xylan, respectively, and stained with Congo Red; panel (A,B) lane 2: protein molecular weight marker; panel (A,B) lane 3: 80  $\mu$ g of total protein. Putative cellulases and xylanases identified in each gel band are listed in Tables 3 and 4, respectively.

Tables 3 and 4 report, among the confidently identified proteins, only those that, on the basis of the annotation in the databases, could possibly be involved in the hydrolysis of cellulose and xylan. Only in three out of the four bands that were positive at the xylanase activity assay (Figure 7B) were enzymes endowed with possible xylanase activity identified.

Several putative cellulases and xylanases were identified. The fact that some of them were detected in more than one protein band could be due to possible different post-translational modifications (fungal extracellular proteins are often variously glycosylated), which might also contribute to a nonperfect separation of proteins in the gel, thus determining a carry-over effect.

#### 4. Conclusions

Thirty-two fungal strains belonging to the genera *Lentinus*, *Pleurotus*, *Trametes* and *Aspergillus* spp. were analyzed for their cellulolytic and xylanolytic abilities. The best enzyme producer *Aspergillus niger* LPB-334 was shown to be able to colonize brewer's spent grain (BSG) by solid state fermentation (SSF) without any addition of nutrients and to produce cellulase and xylanase enzymes. The cellulase- and xylanase-containing extracts were preliminarily characterized for optimal activity as a function of temperature, pH and thermoresistance. Proteins possibly endowed with cellulase and xylanase activity were identified by a proteomic approach and on the basis of database annotation. These are good potential candidates as biocatalysts for BSG valorization and re-use for the production of high-value enzymes to be used for saccharification of wastes and bioenergy production.

**Author Contributions:** Investigation, R.L., A.P., A.D.C.; data curation, L.P.d.S.V., L.B.; writing—original draft preparation, V.F.; writing—review and editing, L.P.d.S.V., L.B., C.R.S.; supervision, V.F., L.P.d.S.V., L.B.; funding acquisition, V.F. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by a Marie Curie International Research Staff Exchange Scheme Fellowship within the 7th European Community Framework Programme: "Improvement of technologies and tools, e.g., biosystems and biocatalysts, for waste conversion to develop an assortment of high added value eco-friendly and cost-effective bio-products" BIOASSORT (contract number 318931).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data supporting the results reported in this article can be found in the tables and figures.

**Acknowledgments:** Part of this research was included in a PhD thesis on Biotechnological Sciences—XXVIII cycle of University of Naples Federico II with the title "Development of new biosystems and biocatalysts for waste upgrading into the high added value products bioethanol and lactic acid" by Rossana Liguori (supervisor Vincenza Faraco).

**Conflicts of Interest:** The authors declare that there is no conflict of interests regarding the publication of this paper.

#### Abbreviations

BSG	brewer's spent grain
SSF	solid state fermentation
CMC	carboxymethylcellulose
BSA	bovine serum albumin
DNS	dinitros-licylic acid reagent

#### References

1. Corrado, S.; Sala, S. Bio-Economy Contribution to Circular Economy. *Des. Sustain. Technol. Prod. Policies* **2018**, *3*, 49–59. [[CrossRef](#)]
2. Liguori, R.; Amore, A.; Faraco, V. Waste valorization by biotechnological conversion into added value products. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6129–6147. [[CrossRef](#)] [[PubMed](#)]
3. Liguori, R.; Faraco, V. Biological processes for advancing lignocellulosic waste biorefinery by advocating circular economy. *Biores. Technol.* **2016**, *215*, 13–20. [[CrossRef](#)] [[PubMed](#)]
4. Ahorsu, R.; Medina, F.; Constantí, M. Significance and Challenges of Biomass as a Suitable Feedstock for Bioenergy and Biochemical Production: A Review. *Energies* **2018**, *11*, 3366. [[CrossRef](#)]

5. Gavrilesco, M.; Gupta, V.G.; Tuohy, M.; Kubicek, C.P.; Saddler, J.; Xu, F. Biorefinery systems: An overview. *Bioenergy Res. Adv. Appl.* **2014**, 219–241. [[CrossRef](#)]
6. Nanda, S.; Mohammad, J.; Reddy, S.N.; Kozinski, J.A.; Dalai, A.K. Pathways of lignocellulosic biomass conversion to renewable fuels. *Biomass Convers. Biorefin.* **2014**, 4, 157–191. [[CrossRef](#)]
7. Capolupo, L.; Faraco, V. Green methods of lignocellulose pretreatment for biorefinery development. *Appl. Microbiol. Biotechnol.* **2016**, 100, 9451–9467. [[CrossRef](#)]
8. Placido, J.; Capareda, S. Ligninolytic enzymes: A biotechnological alternative for bioethanol production. *Bioresour. Bioprocess.* **2015**, 2, 23. [[CrossRef](#)]
9. Den, W.; Sharma, V.K.; Lee, M.; Nadadur, G.; Varma, R.S. Lignocellulosic Biomass Transformations via Greener Oxidative Pretreatment Processes: Access to Energy and Value-Added Chemicals. *Front. Chem.* **2018**, 6, 141. [[CrossRef](#)]
10. Arranz, J.; Sepúlveda, F.; Montero, I.; Romero, P.; Miranda, M. Feasibility Analysis of Brewers' Spent Grain for Energy Use: Waste and Experimental Pellets. *Appl. Sci.* **2021**, 11, 2740. [[CrossRef](#)]
11. Mussatto, S.; Dragone, G.; Roberto, I. Brewers' spent grain: Generation, characteristics and potential applications. *J. Cereal Sci.* **2006**, 43, 1–14. [[CrossRef](#)]
12. Liguori, R.; Soccol, C.R.; Vandenberghe, L.P.D.S.; Woiciechowski, A.L.; Faraco, V. Second Generation Ethanol Production from Brewers' Spent Grain. *Energies* **2015**, 8, 2575–2586. [[CrossRef](#)]
13. Liguori, R.; Soccol, C.R.; Vandenberghe, L.; Woiciechowski, A.; Ionata, E.; Marcolongo, L.; Faraco, V. Selection of the Strain *Lactobacillus acidophilus* ATCC 43121 and Its Application to Brewers' Spent Grain Conversion into Lactic Acid. *BioMed. Res. Int.* **2015**, 2015, 1–9. [[CrossRef](#)] [[PubMed](#)]
14. Amore, A.; Pepe, O.; Ventorino, V.; Birolo, L.; Giangrande, C.; Faraco, V. Industrial waste based compost as a source of novel cellulolytic strains and enzymes. *FEMS Microbiol. Lett.* **2012**, 339, 93–101. [[CrossRef](#)]
15. Florencio, C.; Couri, S.; Farinas, C.S. Correlation between Agar Plate Screening and Solid-State Fermentation for the Prediction of Cellulase Production by *Trichoderma* Strains. *Enzym. Res.* **2012**, 2012, 1–7. [[CrossRef](#)]
16. Montibeller, V.W.; Vandenberghe, L.P.D.S.; Amore, A.; Soccol, C.R.; Birolo, L.; Vinciguerra, R.; Salmon, D.N.X.; Spier, M.R.; Faraco, V. Characterization of Hemicellulolytic Enzymes Produced by *Aspergillus niger* NRRL 328 under Solid State Fermentation on Soybean Husks. *Bioresources* **2014**, 9, 7128–7140. [[CrossRef](#)]
17. Ghose, T.K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, 59, 257–268. [[CrossRef](#)]
18. Bailey, M.J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **1992**, 23, 257–270. [[CrossRef](#)]
19. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, 31, 426–428. [[CrossRef](#)]
20. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, 227, 680–685. [[CrossRef](#)]
21. Amore, A.; Faraco, V. Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production. *Renew. Sustain. Energy Rev.* **2012**, 16, 3286–3301. [[CrossRef](#)]
22. Amore, A.; Parameswaran, B.; Kumar, R.; Birolo, L.; Vinciguerra, R.; Marcolongo, L.; Ionata, E.; Lacara, F.; Pandey, A.; Faraco, V. Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR44A in brewer's spent grain saccharification. *J. Chem. Technol. Biotechnol.* **2014**, 90, 573–581. [[CrossRef](#)]
23. Lamb, J.; Loy, T. Seeing red: The use of Congo Red dye to identify cooked and damaged starch grains in archaeological residues. *J. Archaeol. Sci.* **2005**, 32, 1433–1440. [[CrossRef](#)]
24. Ang, T.N.; Ngoh, G.C.; Chua, A.S.M. A quantitative method for fungal ligninolytic enzyme screening studies. *Asia Pac. J. Chem. Eng.* **2010**, 6, 589–595. [[CrossRef](#)]
25. Kang, S.W.; Park, Y.S.; Lee, J.S.; Hong, S.I.; Kim, S.W. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Biores. Technol.* **2004**, 91, 153–156. [[CrossRef](#)]
26. Ncube, T.; Howard, R.L.; Abotsi, E.K.; van Rensburg, E.L.J.; Ncube, I. *Jatropha curcas* seed cake as substrate for production of xylanase and cellulase by *Aspergillus niger* FGSCA733 in solid-state fermentation. *Ind. Crop. Prod.* **2012**, 37, 118–123. [[CrossRef](#)]
27. Ang, S.K.; Shaza, E.M.; Adibah, Y.A.; Suraini, A.A.; Madihah, M.S. Production of cellulases and xylanase by *Aspergillus fumigatus* SK1 using untreated oil palm trunk through solid state fermentation. *Process Biochem.* **2013**, 48, 1293–1302. [[CrossRef](#)]
28. da Silva, P.D.; Pirotta, R.D.P.B.; Codimaa, C.A.; Tremacoldi, C.R.; Rodrigues, A.; Farinas, C.S. Effect of initial moisture content on two Amazon rainforest *Aspergillus* strains cultivated on agro-industrial residues: Biomass-degrading enzymes production characterization. *Ind. Crop. Prod.* **2013**, 42, 236–242. [[CrossRef](#)]
29. Bhavsar, K.; Kumar, V.R.; Khire, J.M. High level phytase production by *Aspergillus niger* NCIM 563 in solid state culture: Response surface optimization, up-scaling, and its partial characterization. *J. Ind. Microbiol. Biotechnol.* **2011**, 38, 1407–1417. [[CrossRef](#)]
30. Dai, X.-J.; Liu, M.-Q.; Jin, H.-X.; Jing, M.-Y. Optimisation of solid-state fermentation of *Aspergillus niger* JL-15 for xylanase production and xylooligosaccharides preparation. *Czech J. Food Sci.* **2011**, 29, 557–567. [[CrossRef](#)]
31. Rodríguez-Fernández, D.; Rodríguez-León, J.; de Carvalho, J.; Sturm, W.; Soccol, C.R. The behavior of kinetic parameters in production of pectinase and xylanase by solid-state fermentation. *Bioresour. Technol.* **2011**, 102, 10657–10662. [[CrossRef](#)]



32. Rodríguez-Zúñiga, U.F.; Neto, V.B.; Couri, S.; Crestana, S.; Farinas, C.S. Use of Spectroscopic and Imaging Techniques to Evaluate Pretreated Sugarcane Bagasse as a Substrate for Cellulase Production Under Solid-State Fermentation. *Appl. Biochem. Biotechnol.* **2014**, *172*, 2348–2362. [[CrossRef](#)]
33. Sanchez, S.M.; Sanchez, I.G.; Arevalo-Villena, M.; Perez, A.B. Production and immobilization of enzymes by solid-state fermentation of agroindustrial waste. *Bioprocess Biosyst. Eng.* **2015**, *38*, 587–593. [[CrossRef](#)]
34. Abrão, F.O.; Duarte, E.R.; Pessoa, M.S.; Dos Santos, V.L.; Júnior, L.F.D.F.; Barros, K.D.O.; Hughes, A.F.D.S.; Silva, T.D.; Rodriguez, N.M. Notable fibrolytic enzyme production by *Aspergillus* spp. isolates from the gastrointestinal tract of beef cattle fed in lignified pastures. *PLoS ONE* **2017**, *12*, e0183628. [[CrossRef](#)]
35. Yang, P.; ZhangLili, H.; Zheng, C.; Jiang, S. Construction of *Aspergillus niger* integrated with cellulase gene from *Ampullaria gigas* Spix for improved enzyme production and saccharification of alkaline-pretreated rice straw. *3 Biotech* **2016**, *6*, 236. [[CrossRef](#)] [[PubMed](#)]
36. Sandhu, S.K.; Oberoi, H.; Babbar, N.; Miglani, K.; Chadha, B.S.; Nanda, D. Two-Stage Statistical Medium Optimization for Augmented Cellulase Production via Solid-State Fermentation by Newly Isolated *Aspergillus niger* HN-1 and Application of Crude Cellulase Consortium in Hydrolysis of Rice Straw. *J. Agric. Food Chem.* **2013**, *61*, 12653–12661. [[CrossRef](#)]
37. Lee, C.K.; Darah, I.; Ibrahim, C.O. Production and optimization of cellulose enzyme using *Aspergillus niger* USM AI 1 and comparison with *Trichoderma reesei* via solid state fermentation system. *Biotechnol. Res. Int.* **2011**, *2011*, 658493. [[CrossRef](#)] [[PubMed](#)]
38. Mrudula, S.; Murugammal, R. Production of cellulase by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. *Braz. J. Microbiol.* **2011**, *42*, 1119–1127. [[CrossRef](#)]
39. Bansal, N.; Tewari, R.; Soni, R.; Soni, S.K. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Manag.* **2012**, *32*, 1341–1346. [[CrossRef](#)] [[PubMed](#)]
40. Umsza-Guez, M.A.; Díaz, A.B.; De Ory, I.; Blandino, A.; Gomes, E.; Caro, I. Xylanase production by *Aspergillus awamori* under solid state fermentation conditions on tomato pomace. *Braz. J. Microbiol.* **2011**, *42*, 1585–1597. [[CrossRef](#)] [[PubMed](#)]
41. Amir, A.; Arif, M.; Pande, V. Purification and characterization of xylanase from *Aspergillus fumigatus* isolated from soil. *Afr. J. Biotechnol.* **2013**, *12*, 3049–3057.
42. Abbas, A.A. Comparative study for production and characterization of D-Xylanase produced from locally isolate *Aspergillus niger* by submerged and solid state fermentation. *J. Int. Environ. Appl. Sci.* **2016**, *11*, 189–197.
43. Pham, T.H. Cloning, Expression, Purification, and Properties of an Endoglucanase Gene (Glycosyl Hydrolase Family 12) from *Aspergillus niger* VTCC-F021 in *Pichia pastoris*. *J. Microbiol. Biotechnol.* **2011**, *21*, 1012–1020. [[CrossRef](#)] [[PubMed](#)]
44. Lee, Y.-J.; Kim, B.-K.; Lee, B.-H.; Jo, K.-I.; Lee, N.-K.; Chung, C.-H.; Lee, Y.-C.; Lee, J.-W. Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. *Bioresour. Technol.* **2008**, *99*, 378–386. [[CrossRef](#)] [[PubMed](#)]
45. Bajaj, P.; Mahajan, R. Cellulase and xylanase synergism in industrial biotechnology. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 8711–8724. [[CrossRef](#)] [[PubMed](#)]