

Article

Production of Xylanase and Cellulase by Two Isolated *Aspergillus* Strains Through Solid-State Fermentation of Lignocellulosic Residues

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Abstract

Seventy-five thermophilic fungi capable of growing at 45 °C were isolated from sugarcane bagasse samples and cultivated under solid-state fermentation (SSF). Two isolates showing the highest xylanase and cellulase production were selected and identified as *Aspergillus fumigatus* 5S2 and *Aspergillus* sp. 1.6C. Water hyacinth (WH) and sugarcane bagasse (SCB) were evaluated as lignocellulosic supports and carbon sources for enzyme production under SSF. Eight culture conditions were evaluated to identify the most suitable condition for enzyme production. Results revealed that substrate recalcitrance was a major limiting factor for enzyme production and that thermochemical pretreatment significantly improved substrate utilization. The highest enzyme activities were achieved on pretreated WH (PWH) supplemented with NaNO₃ and mineral salts. *A. fumigatus* 5S2 reached 810 U/g dry matter (DM) for xylanase and 95 U/g DM for cellulase, whereas *Aspergillus* sp. 1.6C reached 649 U/g DM of xylanase and 33 U/g DM of cellulase under the corresponding optimal culture conditions. PWH was mixed with SCB to improve the physical properties of the SSF support, including aeration, porosity, and water retention. The highest enzyme production levels were obtained using 1:1 and 1:0.5 (*w/w*) mixtures of PWH and SCB for *A. fumigatus* 5S2 and *Aspergillus* sp. 1.6C, respectively. SSF column bioreactors packed with these mixtures produced similar enzyme titers at 30 °C and 40 °C, confirming the thermotolerant nature of both strains.

Keywords: water hyacinth; sugarcane bagasse; solid-state fermentation; xylanase and cellulase production; thermotolerant fungi



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1. Introduction

Lignocellulosic biomass is the most sustainable and abundant renewable carbon source for production of high-value bioproducts. Among the diverse range of available feedstocks,

sugarcane bagasse and water hyacinth (*Eichhornia crassipes*) stand out as promising substrates for cellulolytic and hemicellulolytic enzyme production due to their carbohydrate composition and worldwide availability.

Sugarcane bagasse (SCB), the fibrous residue remaining after juice extraction, is one of the largest agro-industrial byproducts generated worldwide, with an estimated annual production of 540–585 million tons [1]. Conversely, water hyacinth (WH) is a fast-growing invasive aquatic macrophyte native to the Amazon Basin in South America, which has spread worldwide and causes significant ecological problems by clogging waterways and reducing oxygen availability in aquatic ecosystems. *Eichhornia crassipes* is considered one of the 100 most damaging invasive alien species due to its rapid proliferation, reaching biomass productivities of 60 to 100 tons per hectare per year [2].

The effective conversion of both lignocellulosic biomasses into value-added products requires the degradation of their main structural polymers, cellulose and hemicellulose. While cellulose consists of glucose units linked through β -1,4-glycosidic bonds, hemicellulose is formed by a heterogeneous matrix of pentoses (xylose and arabinose), hexoses (glucose, mannose, and galactose), and sugar acids [3,4]. The degradation of these polysaccharides requires the coordinated action of cellulolytic and hemicellulolytic enzymes. Among the hemicellulolytic enzymes, xylanases are primarily responsible for xylan hydrolysis. Specifically, xylanases (β -1,4-endoxylanases EC 3.2.1.8) hydrolyze the internal glycosidic bonds present in xylan. On the other hand, cellulase is a collective term that includes endoglucanases, exoglucanases, and β -glucosidases. Endoglucanases (EC 3.2.1.4) hydrolyze β -1,4 glycosidic bonds within cellulose chains, releasing oligomers. Exoglucanases (EC 3.2.1.91) further degrade these oligomers by releasing soluble cellobiose from the ends of the chains, whereas β -glucosidases (EC 3.2.1.21) hydrolyze cellobiose into glucose [5].

Evaluating the production of both xylanases and cellulases by some microorganisms provides a direct indicator of their capacity to disrupt and valorize these specific plant cell walls. Among microbial producers, fungi are recognized as highly efficient lignocellulolytic enzyme producers [6]. In addition to their adaptability to diverse substrates, filamentous fungi can be cultivated on low-cost agro-industrial residues, particularly through solid-state fermentation (SSF), making them attractive organisms for industrial enzyme production [7].

Thermophilic and thermotolerant fungi are known for their ability to produce a wide array of enzymes, such as amylases, proteases, lipases, phosphatases, xylanases, pectinases, laccases, cellulases, hemicellulases, and glycoside hydrolases, which often exhibit higher catalytic efficiency than their mesophilic counterparts [8,9]. These fungi generally show optimal growth between 45 and 50 °C, with maximum growth temperatures below 62 °C. Both groups are distinguished based on their minimum growth temperature: thermophilic fungi have a minimum at or above 20 °C, whereas thermotolerant fungi can grow well below this value [10].

Because thermophilic fungal enzymes possess inherent thermostability and resistance to adverse physicochemical conditions, including tolerance to chemical compounds and metal ions that may inhibit enzyme activity, thermostable lignocellulolytic enzymes have received considerable attention due to their potential applications in several industrial processes, such as pulp bleaching in the paper and pulp industry, animal feed production, and bread and beverage processing [11,12].

A. fumigatus is a thermotolerant filamentous fungus capable of efficiently degrading plant cell wall lignocellulose. This species exhibits efficient growth and produces cellulases and xylanases when cultivated on agro-industrial residues and other lignocellulosic materials [13].

Among the available bioprocesses, SSF has emerged as an attractive strategy for enzyme production because of its operational simplicity, low water requirement, and suitability for fungal growth on lignocellulosic substrates [12,14]. However, one of the main challenges associated with SSF is the difficulty in controlling process parameters, particularly temperature, due to limited heat dissipation within the solid matrix [15]. This often generates heterogeneous conditions that may affect microbial growth and enzyme synthesis. Therefore, the selection of microorganisms capable of tolerating thermal fluctuations becomes critical.

In this context, thermotolerant fungi represent promising biological systems for SSF processes, since they are able to grow and maintain metabolic activity over a broad temperature range. This characteristic could help address one of the major challenges associated with temperature control in SSF systems [16].

Despite these advantages, only a limited number of studies have systematically evaluated the combined effect of substrate characteristics and incubation temperature on enzyme production by thermotolerant fungi under SSF conditions. In particular, the influence of different lignocellulosic substrates on enzymatic performance remains insufficiently understood. Additionally, alternative and underutilized biomass sources, such as water hyacinth, an invasive aquatic plant with high lignocellulosic content, have been scarcely explored in this context.

The aim of this study was to evaluate the potential of two lignocellulosic residues, water hyacinth and sugarcane bagasse, as supports and carbon sources for xylanase and cellulase production by thermotolerant filamentous fungi under solid-state fermentation. To achieve this objective, thermotolerant fungi isolated from sugarcane bagasse were screened to identify the most promising enzyme producers, and the effects of lignocellulosic support, thermochemical pretreatment, nitrogen source, and support formulation on enzyme production were investigated. Finally, the performance of the selected strains was evaluated in column bioreactors operated at two temperatures to assess their suitability for scalable SSF processes.

2. Materials and Methods

2.1. Sampling and Characterization of Vegetal Biomasses (Water Hyacinth and Sugarcane Bagasse)

Water hyacinth (WH) plants were harvested from Las Pintas lagoon (Tlaquepaque, Jalisco, Mexico; 20°34′43.7″ N 103°19′50.2″ W) and sectioned into roots, stems and leaves. The stems were selected for use as the solid support and carbon source for enzyme production under solid-state fermentation (SSF). Sugarcane bagasse (SCB) was supplied by Compañía Azucarera del Ingenio Bellavista S.A. de C.V. (Bellavista, Acatlán de Juárez, Jalisco, Mexico (20°26′09.8″ N 103°37′60.0″ W)).

WH plants were rinsed with tap water to remove adhering impurities, whereas SCB was washed twice with tap water and once with distilled water in batch mode to reduce residual sugars and remove surface contaminants. Both materials were then dried at 70 °C for 72 h in a convection oven (Terlab, Zapopan, Mexico), milled using a blade mill (Vayco, Mexico), and sieved to obtain three particle size fractions: coarse (0.84–2.00 mm) used for non-pretreated biomasses for SSF; medium (0.42–0.84 mm) used to prepare pretreated biomasses for SSF; and fine (≤ 0.42 mm) used for inoculum preparation and preservation media.

2.2. Isolation of Thermophilic Fungal Strains

Thermophilic fungal strains were isolated from SCB samples collected at the “Ingenio El Higo” sugar mill, located in El Higo, Veracruz, Mexico (21°46′24.5″ N 98°27′18.2″ W). This SCB was used exclusively for fungal isolation because of the high ambient temper-

atures (up to 40 °C) at the collection site. In contrast, the SCB used as the lignocellulosic support in all SSF experiments was obtained from Ingenio Bellavista, Jalisco, as described in Section 2.1. SCB fragments were placed directly onto the surface of potato dextrose agar (PDA) supplemented with 100 mg/L chloramphenicol in Petri dishes and incubated in duplicate at 45 °C for 48 h. Colonies exhibiting distinct morphological characteristics were selected and purified by successive transfers onto fresh PDA plates. Axenic cultures were obtained after seven consecutive transfers, each followed by incubation at 45 °C for 48 h.

For preservation, the fungal isolates were cultivated on a solid medium containing (g/L): finely ground (≤ 0.42 mm) WH stems, 10; glucose, 5; $(\text{NH}_4)_2\text{SO}_4$, 4.73; KH_2PO_4 , 1.98; MgSO_4 , 0.51; CaCl_2 , 0.39; NaCl , 0.13; FeSO_4 , 0.05; agar, 15; and 4 mL of a trace element solution. The trace element solution contained (g/L): $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.98; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2.378; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.29; and EDTA, 10.

The pH of the medium was adjusted to 6.5 before sterilization at 121 °C for 15 min. The medium was dispensed into slant tubes, inoculated with fungal spores, and incubated at 30 °C for 48 h. The resulting culture collection, comprising 75 isolates, was stored at 5 °C.

2.3. Inoculum Preparation

Spores were recovered from the slant cultures and used to inoculate the preservation medium. Briefly, 50 mL of medium was dispensed into 250 mL Erlenmeyer flasks, sterilized at 121 °C for 15 min, and inoculated after solidification. The cultures were incubated at 30 °C for 72 h to promote sporulation.

Spores were harvested by adding 25 mL of Tween 80 (0.01% *w/v*), followed by gentle magnetic stirring for 5 min. The resulting suspension was filtered through two layers of sterile gauze to remove mycelial fragments. Spore concentration was determined using a Neubauer counting chamber under an optical microscope at 40 $\times$ magnification.

2.4. Screening of Thermophilic Fungal Strains Producing Xylanases and Cellulases

Xylanase and cellulase production were tested by culturing the 75 isolated fungal strains under SSF, using WH with particle sizes from 0.841 to 2.00 mm as support. The culture medium was based on the preservation medium described above, excluding agar. SSF was carried out as follows: 2 g of dry WH was placed in 250 mL Erlenmeyer flasks and sterilized at 121 °C for 15 min. Separately, the liquid culture medium was sterilized and inoculated to achieve a final concentration of 3×10^7 spores per gram of dry support. Subsequently, 8 mL of the inoculated medium was aseptically added to each flask and thoroughly mixed with the support using a sterile spatula to obtain a final moisture content of 80% (*w/w*).

Fermentations were conducted at 45 °C for 48 h to efficiently identify the best enzyme-producing strains. After incubation, samples were transferred to plastic bags and stored at -20 °C until further analysis. Two fungal strains (1.6C and 5S2) were selected based on their high xylanase and cellulase production.

2.5. Morphological and Molecular Identification of Two Thermophilic Fungal Strains

Two thermophilic fungal isolates (1.6C and 5S2) were preliminarily identified based on their colonial and microscopic morphological characteristics, observed after their incubation at 30 °C on PDA using a Zeiss Primo Star light microscope (Carl Zeiss MicroImaging, Gottingen, Germany) [17].

For molecular identification, both fungal strains were grown in 250 mL Erlenmeyer flasks containing 50 mL of potato dextrose broth at 30 °C and 170 rpm for 48 h. Mycelial biomass was harvested by vacuum filtration on Whatman No. 1 filter paper, rinsed with sterile distilled water, and stored at -20 °C until DNA extraction.

Genomic DNA was isolated from the mycelium using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer protocol. The internal transcribed spacer (ITS1-5.8S-ITS2) region of the rDNA was amplified by PCR using primers ITS1 and ITS4 [18] with OneTaq Hot Start DNA Polymerase (New England Biolabs, Ipswich, MA, USA). PCR amplification was performed with an initial denaturation at 95 °C for 30 s, followed by 35 cycles of 95 °C for 20 s, 55 °C for 30 s, and 68 °C for 1 min, with a final extension at 68 °C for 5 min. Amplicons were purified and submitted for sequencing (Macrogen USA Corp., Rockville, MD, USA). The resulting sequences were assembled with CLC Main Workbench 8.1 (CLCBio, Aarhus, Denmark) and queried against the NCBI database using BLASTn (accessed on 10 June 2024). Taxonomic identification was assigned based on sequence similarity, with $\geq 99\%$ identity considered indicative of species-level assignment and $\geq 97\%$ for genus-level classification. Isolates not meeting these thresholds were conservatively assigned to the lowest supported taxonomic rank.

2.6. Effect of Temperature on Fungal Growth

Both fungal strains were inoculated at the center of potato dextrose agar (PDA) plates using a sterile toothpick carrying fungal spores, which was gently inserted into the agar surface. Radial growth rate ($\mu\text{m}/\text{h}$) was determined by measuring the colony diameter over time at different incubation temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60 °C). All experiments were performed in triplicate.

2.7. Solid-State Fermentations Using Two Vegetal Biomasses as Supports and Two Nitrogen Sources to Improve Xylanases and Cellulases Production

2.7.1. Factorial Screening of Culture Medium Composition

The kinetics of xylanase and cellulase production were performed under SSF for both fungal strains (*A. fumigatus* 5S2 and *Aspergillus* sp. 1.6C) using different culture medium formulations. A full factorial screening design (2^3) was employed to assess the effects of three factors at two levels each: (1) support for SSF (SCB or WH), used simultaneously as lignocellulosic support and primary carbon source for enzyme production; (2) with or without thermochemical pretreatment of the support; and (3) nitrogen source (NaNO_3 or $(\text{NH}_4)_2\text{SO}_4$). The combinations resulted in eight experimental conditions for each strain (Table 1).

Table 1. Culture media used to improve xylanase and cellulase production under SSF.

Combination	Lignocellulosic Support	Thermal Acid Pretreatment	Nitrogen Source
1	SCB	Yes	NaNO_3
2	SCB	Yes	$(\text{NH}_4)_2\text{SO}_4$
3	SCB	No	NaNO_3
4	SCB	No	$(\text{NH}_4)_2\text{SO}_4$
5	WH	Yes	NaNO_3
6	WH	Yes	$(\text{NH}_4)_2\text{SO}_4$
7	WH	No	NaNO_3
8	WH	No	$(\text{NH}_4)_2\text{SO}_4$

Maximum cellulase and xylanase activities expressed in U/g dry matter (DM) obtained during the fermentation kinetics were used to calculate maximum enzyme productivity according to Equation (1).

$$M_{EP(i)} = \frac{\max(EA_{(i)})}{t_{F(i)}} \quad (1)$$

where $M_{EP(i)}$ is the maximum enzyme productivity (U/g DM·h) of cellulase or xylanase activity (U/gDM) along the dynamic fermentation process. The term $\max(EA_{(i)})$ is the identified maximum enzyme activity of each experimental run and $t_{F(i)}$ is the time (h) in which this activity reaches its maximum value.

For non-pretreated supports, the culture medium was identical to that described in Section 2.4; only the nitrogen source concentration was modified to 6.07 g/L when NaNO_3 was used. The pH was adjusted to 6.5 before being sterilized at 121 °C for 15 min.

For pretreated supports, a concentrated version of the same culture medium was used to compensate for the additional moisture introduced during pretreatment and to maintain comparable nutrient availability, considering the presence of residual salts generated during neutralization (Na_2SO_4). The salt solution composition (g/L) was: $(\text{NH}_4)_2\text{SO}_4$, 30.96 or NaNO_3 , 39.83; KH_2PO_4 , 12.92; and MgCl_2 , 1.23. The pH was adjusted to 6.5, prior to sterilization at 121 °C for 15 min. The medium was inoculated to achieve a final concentration of 7.5×10^6 spores per milliliter.

Thermochemical pretreatment of WH and SCB (medium particle size) was performed by impregnation with 0.6 M H_2SO_4 (2 mL/g dry support), followed by autoclaving at 121 °C for 20 min. After cooling, the material was neutralized by adding 1 mL of 2.4 M NaOH /g dry support and subsequently mixed with 1 mL of inoculated medium/g dry support, reaching an inoculum concentration of 7.5×10^6 spores/g dry support. A total of 10 g of the resulting mixture was transferred into 250 mL Erlenmeyer flasks.

SSF experiments were conducted at 45 °C for 96 h under the conditions described above (Section 2.4), unless otherwise specified. The culture period was extended to 96 h because this stage aimed to evaluate enzyme production kinetics and identify the maximum enzyme activity under each experimental condition. Samples (two flasks) were collected every 24 h and stored at −20 °C until further analysis.

2.7.2. Evaluation of Biomass Mixtures Used as Supports for Xylanase and Cellulase Production by SSF

The effect of using biomass mixtures (sugarcane bagasse [SCB] and pretreated water hyacinth [PWH]) as supports for SSF on xylanase and cellulase production was evaluated in 250 mL Erlenmeyer flasks using both fungal strains (*A. fumigatus* 5S2 and *Aspergillus* sp. 1.6C). For each fungal strain, SSF was performed using SCB alone, PWH alone, and two SCB:PWH mixtures (1:1 and 1:0.5, *w/w*). Through preliminary experimental assays, these mixture ratios were selected to provide high enzyme production while maintaining the structural porosity of the SSF support. This porosity is required for adequate bed aeration, as well as for mass and heat transfer and fungal colonization. Mixtures with higher proportions of PWH relative to SCB experienced bed compaction, which caused the culture medium to drain away. Conversely, mixtures with lower proportions of PWH to SCB significantly reduced enzyme production.

SCB and PWH were prepared and impregnated with the culture media described in Section 2.7.1 for non-pretreated (SCB) and pretreated (PWH) supports, respectively. For mixed supports, the culture medium corresponding to non-pretreated supports (Section 2.7.1) was proportionally concentrated to maintain comparable nutrient concentrations and a moisture content of 80% (*w/w*). The concentration factors were determined by mass balance, considering that the PWH in each mixture incorporates the residual liquid resulting from the consecutive addition of H_2SO_4 (0.6 M, 2 mL/g dry matter) and NaOH (2.4 M, 1 mL/g dry matter), during the thermochemical pretreatment step. Therefore, a medium that was 1.6 times more concentrated was used for the 1:1 (SCB:PWH) mixture, and a medium that was 1.3 times more concentrated was used for the 1:0.5 (SCB:PWH) mixture.

SCB, PWH, and the corresponding inoculated culture media were thoroughly mixed to ensure uniform distribution. A total of 10 g of each mixture was transferred into a 250 mL Erlenmeyer flask and incubated at 30 °C for 72 h. Fermentations were performed in duplicate, and fermented solids were collected after incubation and stored at −20 °C until further analysis.

2.8. Solid-State Fermentation in Column Bioreactors

Based on the flask experiments, the optimal SCB:PWH mixture was selected for each fungal strain to perform kinetic studies in column bioreactors. Fermentations were carried out for 120 h at 30 °C and 40 °C to obtain complete enzyme production profiles under conditions representative of packed-bed SSF.

Glass columns (2.7 cm internal diameter and 32 cm height) were used as bioreactors, each packed with approximately 14 g of the selected support mixture, corresponding to the working volume of the reactor, prepared as described in Section 2.7.2. The columns were placed in a thermostated water bath to maintain temperature control.

Moisture content was maintained by passing air through glass humidifiers containing water. The humidified air was introduced at the base of each column to provide an upward airflow of 20 mL/min through the solid matrix. Two independent column bioreactors were sampled every 24 h. The fermented material was collected and stored at −20 °C until further analysis, and each sample was subsequently analyzed in duplicate for all analytical determinations.

2.9. Analytical Assays

Moisture content was determined by drying 5 g of fermented solid material in a convection oven (Terlab, Zapopan, Mexico) at 100 °C for 24 h.

The pH of the fermented samples was determined by suspending 1 g of fermented solid in 10 mL of distilled water. The suspension was mixed using a vortex for 30 min, and the pH was measured in the resulting liquid phase using a potentiometer (LAQUA, HORIBA Scientific, Kyoto, Japan).

Protein concentration was quantified using the Bradford method [19], with bovine serum albumin (BSA) as the calibration standard.

2.10. Enzymatic Activity Assay

Crude enzyme extracts were prepared by suspending 0.3 g of fermented solid in 1 mL of 0.1 M citrate buffer (pH 5.3) in 2 mL microtubes. The suspensions were vigorously mixed for 1 h using a vortex, followed by centrifugation at 4200× g for 15 min using a Firlabo SW9R centrifuge (Firlabo, Meyzieu, France). The resulting supernatants were recovered and used as enzyme extracts.

Xylanase and cellulase activities were quantified by determining the amount of reducing sugars (RS) released from beechwood xylan and carboxymethyl cellulose (CMC), respectively, using the 3,5-dinitrosalicylic acid (DNS) assay [20]. Reducing sugar concentrations were determined from calibration curves prepared for each assay batch using xylose and glucose as standards for the xylanase and cellulase activity, respectively.

For the assays, 20 µL of appropriately diluted enzyme extract was added to 180 µL of substrate solution (1% w/v beechwood xylan, Megazyme®, or 1% w/v CMC, Sigma-Aldrich, St. Louis, MO, USA) prepared in 0.1 M citrate buffer (pH 5.3). Reaction mixtures were incubated at 50 °C for 10 min and subsequently quenched by immersion in an ice-water bath. Then, 50 µL of 1.6 M NaOH and 250 µL of DNS reagent were added, with vortexing after each addition. The samples were heated in a boiling water bath for 5 min, allowed to cool to room temperature, and diluted with 2 mL of distilled water.

Absorbance was recorded at 540 nm using a spectrophotometer (DR/2010, HACH, Loveland, CO, USA). Control reactions were prepared by replacing the enzyme extract with distilled water. All measurements were performed in triplicate. One unit (U) was defined as the amount of enzyme required to release one micromole of RS (expressed as xylose equivalents for xylanase activity or glucose equivalents for cellulase activity) per minute under the assay conditions.

2.11. Statistical Analysis

All experiments were performed at least in duplicate or triplicate, as indicated in each section, and results are presented as mean $\pm$ standard deviation.

For the factorial screening experiment described in Section 2.7.1, the effects of support type (A), pretreatment (B), nitrogen source (C), and their interactions on maximum enzyme productivity were evaluated by analysis of variance (ANOVA) using a full factorial 2^3 design. Effect sizes were estimated using η^2 (simple eta-squared), representing the proportion of total variance explained by each factor or interaction.

Statistical analyses were performed using R software version 4.5.0, and statistical significance was established at $p < 0.05$.

3. Results and Discussion

3.1. Isolation and Identification of Thermophilic and Thermotolerant Xylanase and Cellulase Producing Fungi

The isolation of fungal strains for xylanase and cellulase production is important for several reasons. First, novel strains may exhibit higher enzyme production and productivity due to their better adaptation to different lignocellulosic substrates. In addition, newly identified xylanases and cellulases may exhibit desirable enzymatic properties relevant for industrial applications, such as different optimal pH and temperature conditions, substrate specificity, thermostability, and stability towards inhibitory ions [21,22].

Seventy-five thermophilic fungal strains, capable of growing at 45 °C, were isolated from SCB samples. Among them, two strains (1.6C and 5S2) were selected based on their high xylanase and cellulase production under SSF conditions (Table S1).

Based on their colonial and microscopic morphology, including the characteristic blue-green colony pigmentation and conidial head structures, together with their thermotolerant growth behavior, both isolates were preliminarily assigned to the genus *Aspergillus*, possibly belonging to the species *fumigatus* [17]. Taxonomic identification was further evaluated by sequencing the ITS1-5.8S-ITS2 rDNA region. BLAST analysis against the NCBI database revealed that the ITS sequences of isolate 1.6C (516 bp) showed 93.87% identity with *Aspergillus fumigatus* strain C13 (accession number OQ830476), whereas strain 5S2 (605 bp) showed 99.82% identity with *Aspergillus fumigatus* isolate HT_OC17_6 (accession number MN634562). Based exclusively on ITS sequence similarity, strain 5S2 was identified as *A. fumigatus*, whereas strain 1.6C was conservatively assigned to *Aspergillus* sp. due to the lower percentage of identity. Both sequences were deposited in GenBank under accession numbers PX410098 for strain 1.6C and PX410091 for strain 5S2.

In addition, a Neighbor-Joining phylogenetic tree was constructed based on the alignment of ITS sequences from several *Aspergillus* species retrieved from the GenBank NCBI database (Figure 1). The phylogenetic analysis grouped strains 1.6C and 5S2 within the *A. fumigatus* clade, clearly separated from closely related species such as *Aspergillus fischeri*, *Aspergillus lentulus*, *Aspergillus udagawae*, and *Aspergillus viridinutans*. The phylogenetic tree was generated using MEGA version 6.0.

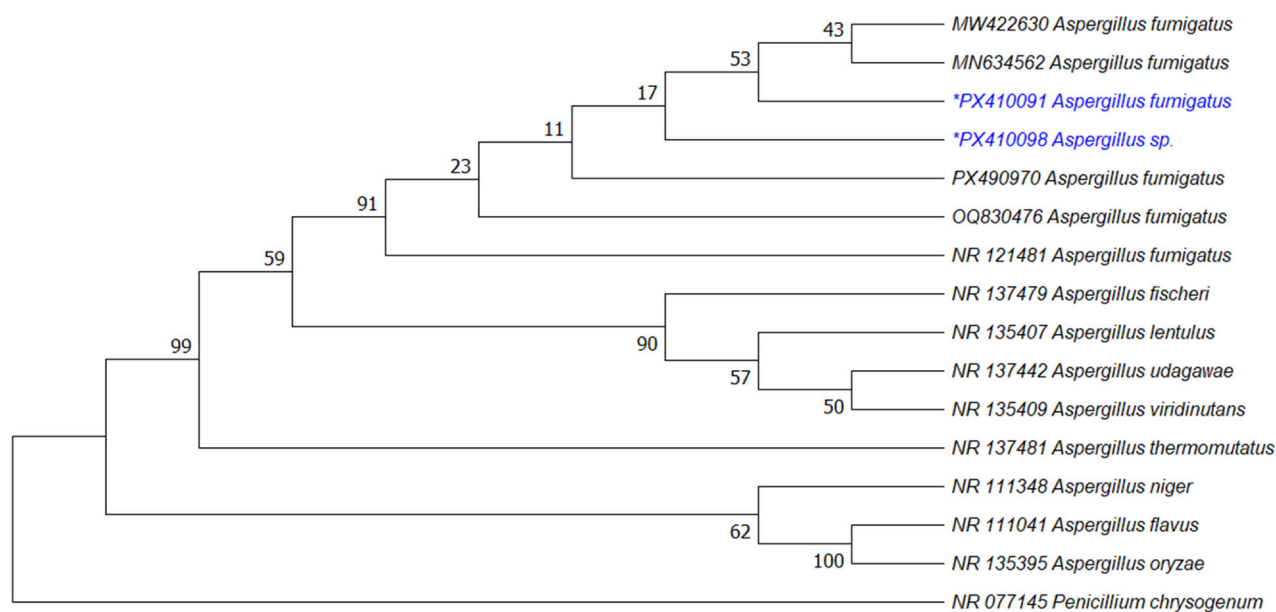


Figure 1. Neighbor-Joining phylogenetic tree based on ITS1-5.8S-ITS2 sequences of *Aspergillus* sp. strain 1.6C (PX410098) and *Aspergillus fumigatus* strain 5S2 (PX410091), compared with sequences from related *Aspergillus* species retrieved from the GenBank NCBI database. *Penicillium chrysogenum* was used as the outgroup. Species names are accompanied by their GenBank accession numbers. Bootstrap values (%) shown next to the branches were calculated from 1000 replicates. * Fungal strains shown in blue are those that were isolated in this study.

A. fumigatus is a saprophytic fungus commonly associated with decaying vegetation [23]. Genomic analyses have shown that this species encodes a wide range of enzymes related to plant biomass degradation [24], which is consistent with the lignocellulolytic activity observed in the present study.

3.2. Radial Growth Rate of Thermotolerant Fungal Strains

In general, thermophilic fungi exhibit optimal growth at elevated temperatures (around 45 °C), whereas thermotolerant fungi are able to grow over a broader temperature range, including lower temperatures (lower than 20 °C). To evaluate the growth-temperature relationship of both fungal isolates, radial growth rates were determined at different incubation temperatures as described in Section 2.6.

Notably, *Aspergillus* sp. 1.6C and *A. fumigatus* 5S2 reached maximum radial growth rates of 714.4 µm/h at 50 °C and 491.6 µm/h at 45 °C, respectively. In addition, the minimum temperatures supporting growth were 5 °C for strain 1.6C and 10 °C for strain 5S2 (Figure 2). This wide growth temperature range permits classification of both strains as thermotolerant.

Other authors have reported maximum radial growth rates for *A. fumigatus* of 394, 550 and 451 µm/h at 30, 40 and 37 °C, respectively, which are comparable to the growth rate observed for strain 5S2 in the present study [25,26]. Differences in radial growth rates between this work and previous reports may be attributed to strain-dependent variability, which influences the ability of the fungus to adapt to different environmental conditions.

3.3. Effect of Culture Medium Composition on Xylanase and Cellulase Production by Thermotolerant Fungal Strains Under Solid-State Fermentation

Since substrate cost plays an important role in the economics of enzyme production, low-value lignocellulosic materials can be used as viable substrates for xylanase and cellulase production; therefore, SSF was selected as the culture method. The effects of support type, thermochemical pretreatment, and nitrogen source on enzyme production by

the selected fungal strains were evaluated under the experimental conditions described in Section 2.7.1.

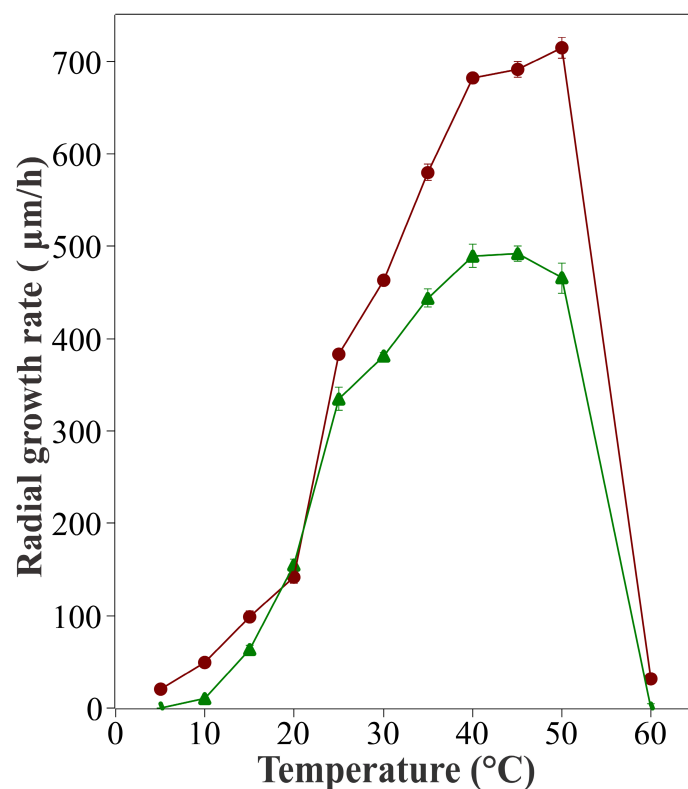


Figure 2. Radial growth rates of thermotolerant fungal isolates *A. fumigatus* 5S2 (▲) and *Aspergillus* sp. 1.6C (●) grown on PDA. Data represent the mean $\pm$ and standard deviation ($n = 3$).

A. fumigatus 5S2 reached maximum xylanase and cellulase activities of 810 and 95 U/g DM, respectively, when grown on pretreated WH supplemented with NaNO_3 as nitrogen source. In contrast, *Aspergillus* sp. 1.6C achieved its maximum xylanase activity (649 U/g DM) under similar conditions but using $(\text{NH}_4)_2\text{SO}_4$, whereas its highest cellulase activity (33 U/g DM) was obtained on non-pretreated SCB supplemented with NaNO_3 (Figures 3 and 4).

The maximum enzyme productivity (M_{EP} , expressed as U/g DM·h) was calculated for all eight evaluated culture conditions using Equation (10). These same MEP values were subsequently subjected to a full factorial ANOVA (2^3 design), whose results are presented in the Supplementary Material (Tables S2–S5), allowing for the identification of the factors that most significantly influence enzyme productivity.

The evaluation of experimental combinations revealed different responses in cellulase and xylanase productivity for strain 5S2. According to Table S2 (Supplementary Material), the factorial ANOVA for cellulase production indicates that all main factors and their interactions significantly affected enzyme productivity ($p < 0.001$). Thermochemical pretreatment (Factor B) was the most influential factor ($\eta^2 = 0.391$), followed by support type (Factor A; $\eta^2 = 0.157$) and the three-way interaction ABC ($\eta^2 = 0.151$). These results indicate that cellulase productivity was influenced not only by the individual factors evaluated but also by their combined effects.

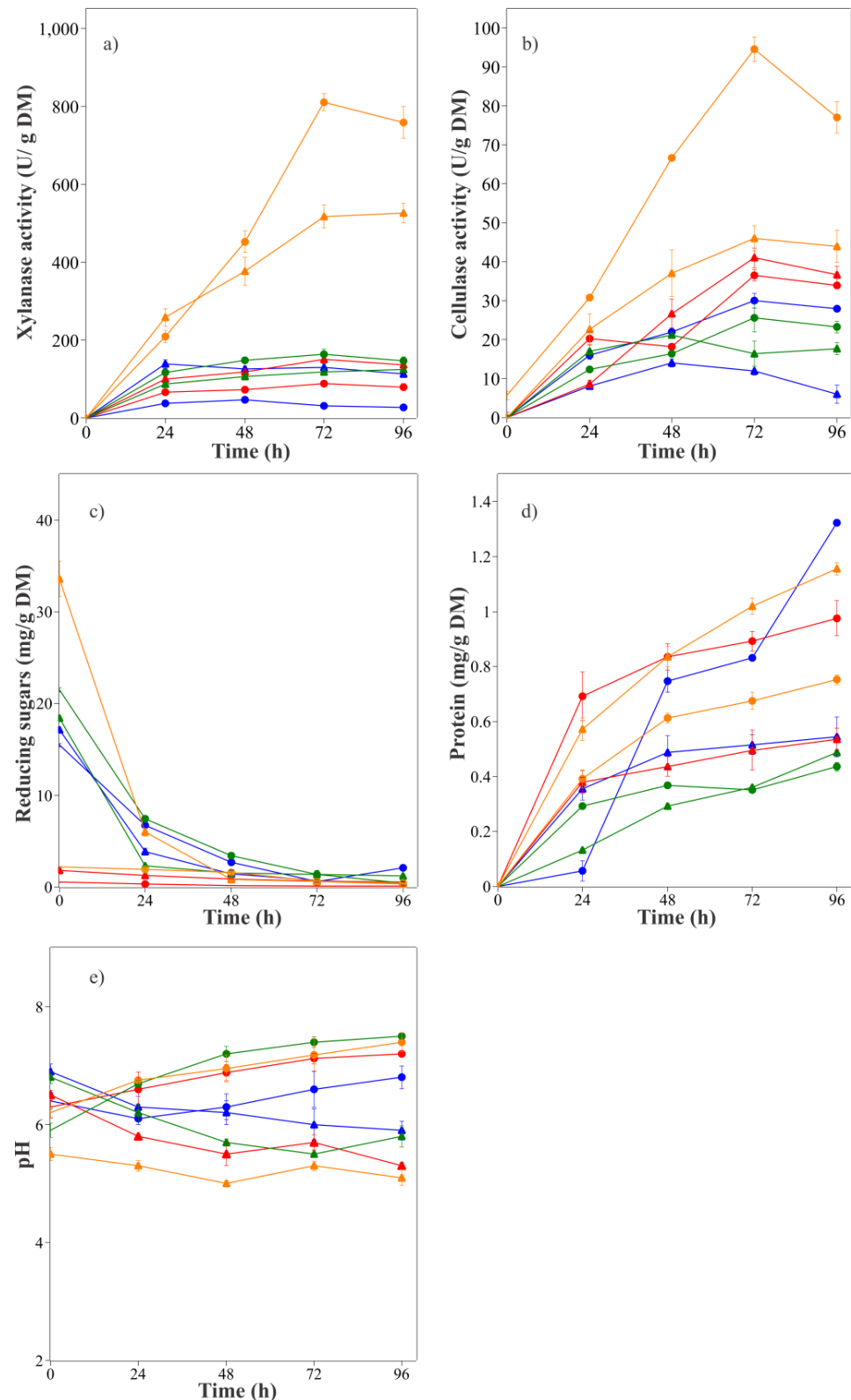


Figure 3. Kinetics of xylanase (a), cellulase (b) and protein (c) production, reducing sugars consumption (d), and pH (e) by *Aspergillus fumigatus* 5S2 under different solid-state fermentation conditions. Eight culture conditions were tested: (1) non-pretreated SCB + NaNO₃ (-●-), (2) non-pretreated SCB + (NH₄)₂SO₄ (-▲-), (3) pretreated SCB + NaNO₃ (-●-), (4) pretreated SCB + (NH₄)₂SO₄ (-▲-), (5) non-pretreated WH + NaNO₃ (-●-), (6) non-pretreated WH + (NH₄)₂SO₄ (-▲-), (7) pretreated WH + NaNO₃ (-●-), and (8) pretreated WH + (NH₄)₂SO₄ (-▲-). Data represent the mean ± standard deviation (n = 3). The error bars are not shown when they are smaller than the symbols.

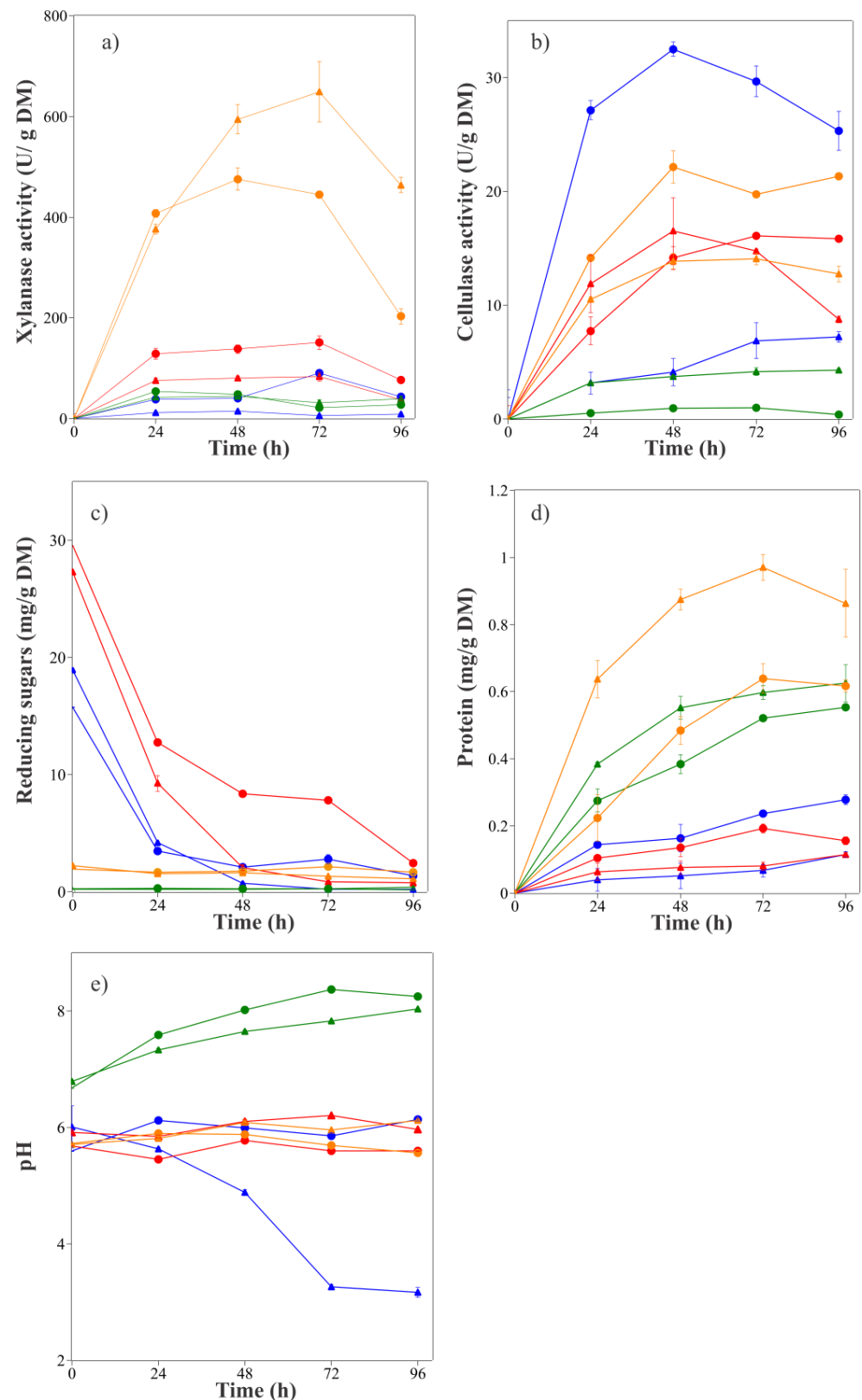


Figure 4. Kinetics of xylanase (a), cellulase (b) and protein (c) production, reducing sugars consumption (d), and pH (e) by *Aspergillus* sp. 1.6C under different solid-state fermentation conditions. Eight culture conditions were tested: (1) non-pretreated SCB + NaNO₃ (-●-), (2) non-pretreated SCB + (NH₄)₂SO₄ (-▲-), (3) pretreated SCB + NaNO₃ (-●-), (4) pretreated SCB + (NH₄)₂SO₄ (-▲-), (5) non-pretreated WH + NaNO₃ (-●-), (6) non-pretreated WH + (NH₄)₂SO₄ (-▲-), (7) pretreated WH + NaNO₃ (-●-), and (8) pretreated WH + (NH₄)₂SO₄ (-▲-). Data represent the mean ± standard deviation ($n = 3$). The error bars are not shown when they are smaller than the symbols.

For xylanase production by strain 5S2, Table S3 (Supplementary Material) shows that support type (Factor A) was the main source of variation ($\eta^2 = 0.371$), followed by thermochemical pretreatment (Factor B; $\eta^2 = 0.2439$). In contrast, nitrogen source (Factor C) did not significantly affect xylanase productivity ($p = 0.4368$). WH consistently promoted higher xylanase productivity than SCB, suggesting that differences in support characteristics influenced enzyme production.

In contrast, strain 1.6C showed a different response pattern. As shown in Table S4 (Supplementary Material), the combined effect of support type, pretreatment, and nitrogen source (interaction ABC) accounted for the largest proportion of the variance ($\eta^2 = 0.344$) for cellulase production. Among the individual factors, nitrogen source was the most influential factor, accounting for 17.0% of the total variance ($\eta^2 = 0.170$). Higher cellulase productivity was observed with NaNO_3 than with $(\text{NH}_4)_2\text{SO}_4$, suggesting that both nitrogen availability and nitrogen form may contribute to enzyme production in this strain.

Conversely, Table S5 (Supplementary Material) reveals that xylanase productivity in strain 1.6C was mainly influenced by support type (Factor A, $\eta^2 = 0.330$) and thermochemical pretreatment (Factor B, $\eta^2 = 0.338$), which jointly explained approximately 72% of the total variance ($p < 0.001$). WH consistently promoted higher xylanase productivity than SCB, although the significant interaction between support type and pretreatment (AB; $\eta^2 = 0.22$) indicated that the effect of thermochemical pretreatment depended on the support used. Although nitrogen source (Factor C) showed a statistically significant effect ($p < 0.001$), its contribution to the total variance was small ($\eta^2 = 0.019$), whereas the remaining interactions (AC, BC, and ABC) were not significant.

Overall, WH promoted higher xylanase and cellulase production than SCB under several of the evaluated conditions. This behavior may be associated with physicochemical characteristics previously reported for water hyacinth, including its relatively lower lignin content than SCB and the presence of proteins and amino acids, although these properties were not directly determined in the present study [27,28]. Additionally, the acid-thermal pretreatment has been reported to improve substrate accessibility, which may have contributed to the enhanced xylanase and cellulase production observed in this study [29]. Similarly, previous studies have shown that physical and chemical pretreatments can enhance enzyme production under SSF. For instance, in SSF processes using SCB and brewer's spent grain, pretreatments such as autoclaving, hot water, or alkaline treatments have been applied to modify substrate structure. These treatments significantly increased xylanase and cellulase production of *Aspergillus niger*, reaching activities of up to 1400 U/g DM and 6.23 U/g DM, respectively, which was attributed to the enhanced accessibility of the structural polysaccharides [30].

However, *Aspergillus* sp. 1.6C exhibited the highest cellulase activity on non-pretreated SCB. This response may be associated with the higher availability of structural cellulose in this substrate, which sustains the constitutive production of cellulases required to initiate cellulose hydrolysis. The resulting cello-oligosaccharides, particularly cellobiose and sophorose, are recognized as the actual inducers of cellulase gene expression in filamentous fungi, thereby promoting further enzyme production in this isolate [31]. Further physicochemical characterization of the substrates would be required to establish the mechanisms underlying this response.

The enzyme activities obtained in the present study are comparable to or higher than those previously reported for filamentous fungi cultured under SSF using lignocellulosic residues [30,32]. Specifically, the xylanase (810 U g⁻¹ DM) and cellulase (95 U g⁻¹ DM) activities obtained with *A. fumigatus* 5S2 are within the upper range of values reported for *A. fumigatus* grown on SSF. For example, *A. fumigatus* SK1 cultivated on untreated

oil palm trunk under SSF produced 418.7 U/g DM of xylanase, along with cellulolytic activities including 54.27 U/g DM of CMCase, 3.36 U/g DM of FPase, and 4.54 U/g DM of β -glucosidase [33]. These findings demonstrate the capacity of *A. fumigatus* to produce coordinated lignocellulolytic enzyme systems on lignocellulosic substrates and support the potential of the thermotolerant isolates evaluated in the present study, for enzyme production under SSF conditions.

3.4. Evaluation of Support Formulation for Scalable Solid-State Fermentation Systems

To develop a culture medium suitable for scale-up in SSF systems such as column or tray bioreactors, the physical properties of the support material must be considered, particularly those related to bed structure, porosity, and moisture retention capacity. These properties are critical for maintaining adequate aeration and preventing liquid leaching from the solid matrix, which may lead to enzyme loss and reduced process efficiency during prolonged fermentation.

Based on the results obtained in the previous stage, pretreated water hyacinth (PWH) promoted high xylanase and cellulase production in both fungal isolates. However, visual inspection of the fermented material indicated a tendency toward compaction, which may compromise its performance in packed-bed systems by limiting interparticle aeration and the space available for fungal hyphae growth.

In this context, coarse SCB particles (0.84–2.00 mm) were incorporated with the aim of improving bed porosity, mechanical stability and moisture retention while ensuring adequate airflow under SSF conditions and providing an additional source of lignocellulosic carbohydrates. Based on the results obtained from the factorial screening, NaNO_3 was selected as the nitrogen source for *A. fumigatus* 5S2, whereas $(\text{NH}_4)_2\text{SO}_4$ was used for *Aspergillus* sp. 1.6C.

For *A. fumigatus* 5S2, the SCB:PWH 1:1 mixture maintained high xylanase production, whereas cellulase activity was lower than that obtained with PWH alone (Figure 5a). Nevertheless, both enzyme activities remained higher than those obtained with the 1:0.5 mixture, indicating that partial substitution of PWH with SCB at an equal proportion preserved a high level of enzyme production while potentially improving the physical properties of the support, as suggested by the reduced tendency toward compaction observed during fermentation. Accordingly, the 1:1 ratio was selected for subsequent column fermentation trials with *A. fumigatus* 5S2.

On the other hand, *Aspergillus* sp. 1.6C exhibited a different pattern of enzyme production (Figure 5b). While PWH alone promoted high enzymatic activities, the SCB:PWH 1:0.5 mixture maintained similar cellulase activity, whereas xylanase activity was higher compared to the other support formulations (only PWH, only SCB and 1:1 mixture). This behavior could be associated with a combined effect of the substrates and with changes in the physical characteristics of the fermentation bed, such as porosity, aeration, and water distribution, which have previously been reported to influence enzyme production under SSF conditions [34]. These results indicate that the 1:0.5 ratio allowed for an enhancement in xylanase production without affecting cellulase activity. Therefore, this formulation was selected for subsequent column SSF experiments with *Aspergillus* sp. 1.6C.

Although the support mixtures were evaluated after the factorial screening, future studies could employ mixture-design methodologies to optimize the relative proportions of lignocellulosic supports (SCB:PWH) over a continuous experimental domain.

3.5. Kinetics of Enzyme Production Using Solid-State Fermentation Performed in Columns

Since both fungal isolates are thermotolerant, with optimal growth temperatures ranging from 35 to 50 °C, studying the effect of incubation temperature on enzyme production

was of particular interest. SSF experiments were performed in column-type bioreactors using the selected SCB:PDH mixtures (1:1 ratio for *A. fumigatus* 5S2 and 1:0.5 ratio for *Aspergillus* sp. 1.6C).

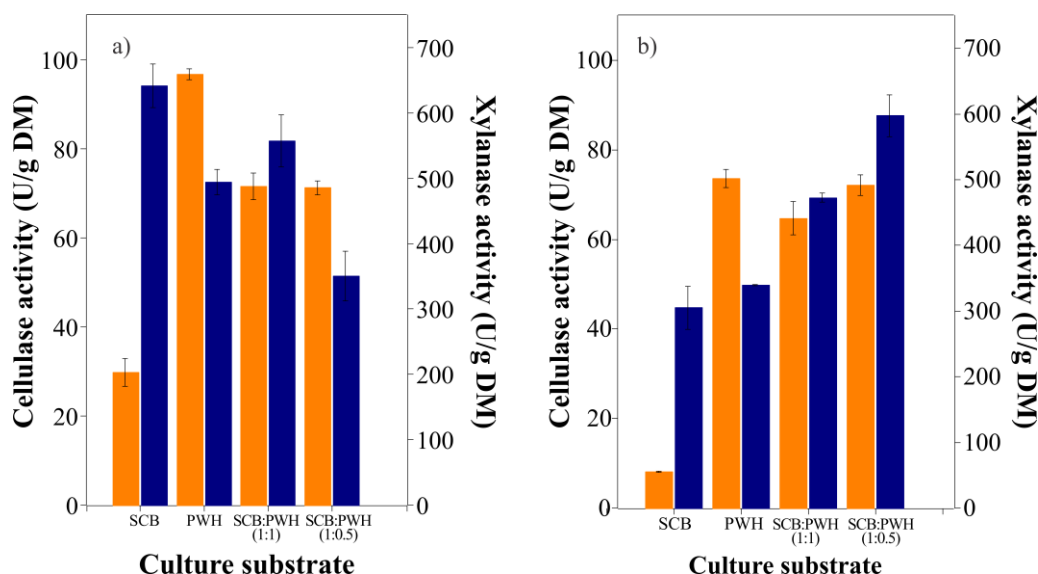


Figure 5. Cellulase (■) and xylanase (■) activities obtained with *A. fumigatus* 5S2 (a) and *Aspergillus* sp. 1.6C (b) after 72 h of SSF in Erlenmeyer flasks, using four support formulations: (1) SCB alone, (2) PDH alone, (3) 1:1 (*w/w*) SCB:PDH mixture and (4) 1:0.5 (*w/w*) SCB:PDH mixture. Data represent mean $\pm$ standard deviation ($n = 3$).

Enzyme production kinetics revealed differences depending on the strain and incubation temperature (Figure 6). Remarkably, both strains exhibited similar maximum enzyme production at 30 and 40 °C (Table 2). However, incubation at 40 °C substantially reduced the time required to reach these maxima. For instance, strain 5S2 reached maximum xylanase production in just 24 h at 40 °C, compared to 120 h at 30 °C, whereas strain 1.6C reached its maximum xylanase production after 72 h and 120 h, respectively. Once peak enzyme production was reached at 40 °C, enzyme activity declined. This result suggests that the enzyme activity may decrease because of reduced stability at 40 °C or may be associated with proteolytic degradation during fungal culture.

Table 2. Maximum enzyme productions and culture times to reach them, in SSF performed at 30 and 40 °C, using column bioreactors packed with mixtures of sugarcane bagasse and pre-treated water hyacinth as supports.

Strain	Culture Temperature	Maximum Xylanase Production (U/g DM)	Time to Reach Maximum Xylanase Production (h)	Maximum Cellulase Production (U/g DM)	Time to Reach Maximum Cellulase Production (h)
<i>Aspergillus</i> sp. 1.6C	30 °C	690.5 $\pm$ 17.3	120	50.7 $\pm$ 2.4	72
	40 °C	708.5 $\pm$ 35.7	72	58.2 $\pm$ 2.3	72
<i>A. fumigatus</i> 5S2	30 °C	424.9 $\pm$ 13.0	120	56.1 $\pm$ 1.2	120
	40 °C	442.5 $\pm$ 10.7	24	55.6 $\pm$ 3.7	72

The utilization of lignocellulosic residue mixtures in SSF has been explored as an approach for improving lignocellulolytic enzyme production, as different materials can provide complementary proportions of cellulose, hemicellulose, lignin, and nutrients. These mixtures may promote fungal growth and the induction of broader enzymatic systems that act synergistically to degrade plant cell wall polysaccharides. For instance, lignocellulosic substrate blends have been shown to enhance cellulase and hemicellulase

production compared to individual substrates [35,36]. Enzymatic activity titers obtained in the present work are highly competitive when compared with previously published reports. For example, *A. niger* and *A. fumigatus* cultivated under SSF using sorghum biomass (SB) combined with wheat bran (WB) produced maximum xylanase activities of 91.8 and 31.4 U/g DM, respectively, using a 1:1 (*w/w*) SB:WB mixture. Under the same conditions, CMCase activities of 29.8 and 27.4 U/g DM were obtained, respectively [37].

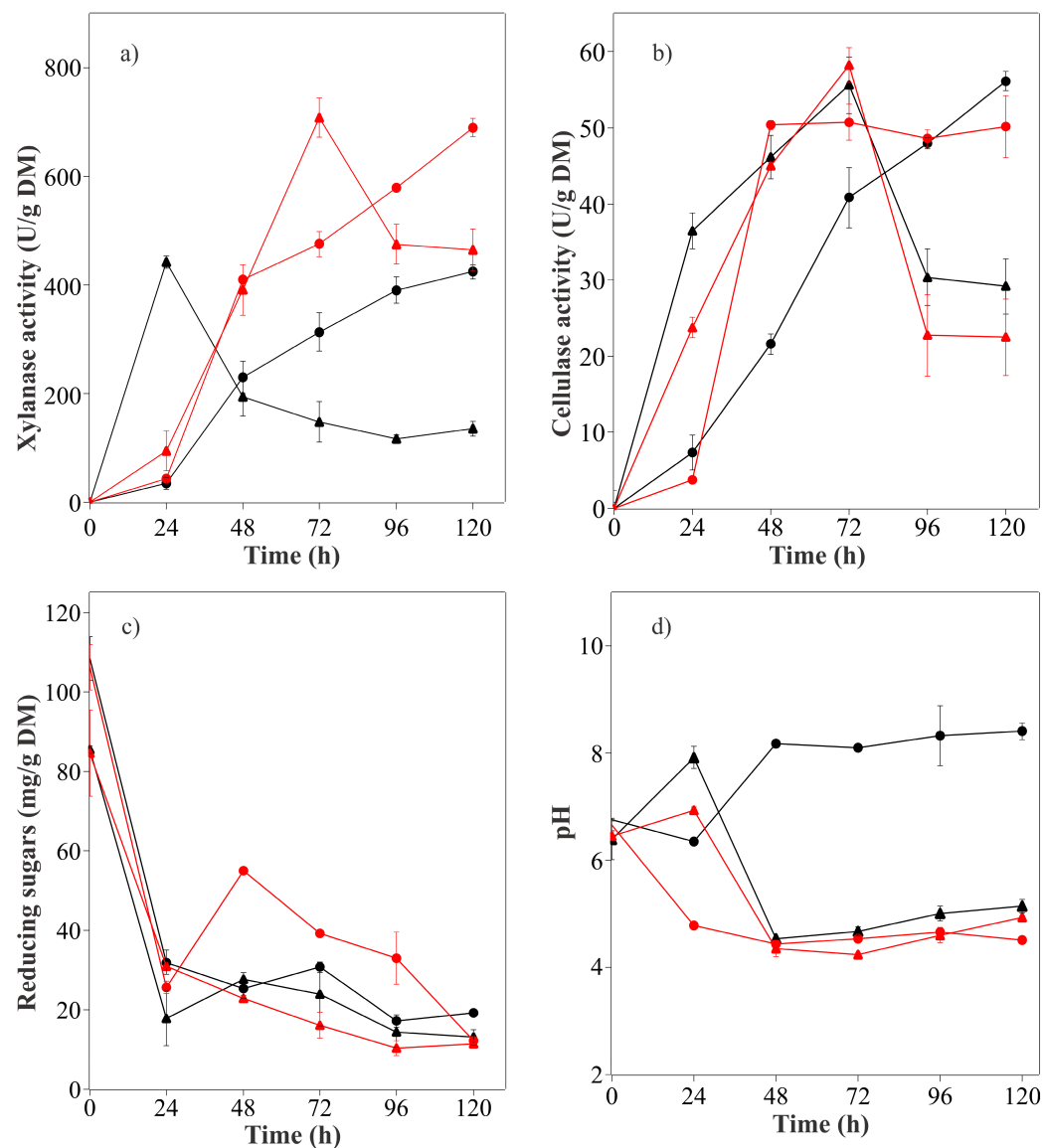


Figure 6. Kinetics of (a) xylanase and (b) cellulase production, (c) reducing sugars, and (d) pH in SSF using column bioreactors. *A. fumigatus* 5S2 was incubated at 30 °C (●) and 40 °C (▲), and *Aspergillus* sp. 1.6C was incubated at 30 °C (●) and 40 °C (▲). Data represent the mean $\pm$ standard deviation of two independent column bioreactors ($n = 2$). Each fermented sample was analyzed in duplicate.

Mixing agro-industrial residues with different structural compositions may improve support porosity and aeration by facilitating airflow among particles, which can enhance fungal colonization and increase substrate accessibility, thereby promoting higher enzymatic production during SSF [38].

In all treatments, reducing sugars decreased sharply within the first 24 h (Figure 6c), indicating rapid substrate consumption during active fungal growth. A transient increase in soluble sugars was observed for *Aspergillus* sp. 1.6C at 30 °C around 48 h,

likely reflecting a temporary imbalance between hydrolysis and sugar assimilation. Subsequently, sugar concentrations declined progressively, consistent with continued uptake and metabolic utilization.

Interestingly, *A. fumigatus* 5S2 exhibited contrasting pH trends at 30 and 40 °C, despite nitrate being used as the nitrogen source in both cases (Figure 6d). These differences may be associated with extracellular pH modulation linked to nitrogen metabolism. In agreement with mechanistic explanations of nitrate reduction and proton balance during nitrogen assimilation, sodium nitrate has been reported to cause medium alkalization in filamentous fungi such as *Trichoderma harzianum*, whereas ammonium typically leads to acidification [39].

Additionally, extracellular pH regulation in fungi can be influenced by the overall metabolic response to temperature, including changes in organic acid secretion and nitrogen assimilation pathways. Nitrogen regulation has been associated with broader shifts in primary and secondary metabolism, including the production of organic acids that lower medium pH [40].

It is worth noting that temperature control is one of the main challenges associated with scaling up an SSF process. The use of thermotolerant fungi could help address this issue by allowing for operation at temperatures commonly used for mesophilic fungi (25–30 °C). Even if the temperature increases during SSF due to metabolic heat release, fungal growth and enzyme production would likely not be substantially affected.

Scale-up of SSF processes remains considerably more challenging than submerged fermentation because heat and mass transfer become increasingly heterogeneous as reactor dimensions increase. Metabolic heat generated during fungal growth may lead to localized temperature gradients, whereas insufficient aeration can reduce oxygen availability and carbon dioxide removal. In addition, uneven moisture distribution throughout the solid bed may affect fungal colonization, substrate accessibility, and enzyme production. Consequently, maintaining uniform environmental conditions within packed-bed bioreactors remains one of the major technological challenges for industrial SSF processes.

In this context, the thermotolerant nature of the isolates evaluated in this work may represent an important advantage for SSF scale-up. Both strains maintained comparable enzyme production at 30 and 40 °C in column bioreactors, despite the different incubation temperatures. This suggests that moderate temperature fluctuations may not substantially affect their enzymatic performance. Furthermore, the incorporation of coarse sugarcane bagasse into the support matrix was intended to improve bed structure and facilitate air circulation, characteristics that could contribute to more robust operation in larger SSF systems [41]. Nevertheless, additional studies at the pilot scale will be required to evaluate oxygen transfer, heat dissipation, moisture gradients, and reactor hydrodynamics under industrial operating conditions.

Although the thermotolerant characteristics of the *A. fumigatus* isolates make them attractive candidates for SSF processes, particularly because of their ability to tolerate temperature fluctuations caused by metabolic heat generation, it is important to recognize that *A. fumigatus* is an opportunistic human pathogen. Therefore, any future industrial implementation should incorporate appropriate biosafety measures, including the use of closed bioreactor systems, control of airborne spore dissemination, and adequate occupational safety practices [42]. Under these conditions, the thermotolerant nature of these isolates may still represent a valuable technological advantage for large-scale SSF processes.

The enzymatic flexibility demonstrated by the two *Aspergillus* sp. isolates in this work, linked to their thermal tolerance and their capacity to utilize two distinct vegetal materials opens the door to utilizing a wider range of lignocellulosic biomasses, establishing this SSF platform as a highly versatile strategy for industrial-scale enzyme production.

4. Conclusions

A total of 75 thermotolerant and thermophilic filamentous fungal strains were isolated from sugarcane bagasse samples. Two of these strains exhibited the highest potential for cellulolytic and xylanolytic production under SSF. Based on morphological and molecular analyses, strain 5S2 was identified as *Aspergillus fumigatus*, whereas strain 1.6C was conservatively assigned to *Aspergillus* sp. Both isolates exhibited thermotolerant growth behavior.

The evaluation of different culture media formulations demonstrated that both substrate (water hyacinth and sugarcane bagasse) selection and thermochemical pretreatment strongly influenced enzyme production. In particular, PWH proved to be an effective lignocellulosic substrate for xylanase and cellulase production, highlighting the potential of this invasive aquatic plant as a low-cost feedstock for enzyme production and biomass valorization.

Pretreating WH resulted in a more compact support matrix during fermentation, based on visual observations. Therefore, mixing PWH with SCB improved the physical characteristics of the fermentation support while maintaining high enzyme production. These mixed supports represent a practical alternative for SSF systems in which adequate aeration, moisture retention, and bed stability are required.

Finally, SSF performed in column bioreactors showed that both strains maintained similar enzyme production at 30 and 40 °C, confirming their thermotolerant nature and suggesting that these isolates could help mitigate some of the temperature-control challenges commonly encountered during SSF scale-up. Although *A. fumigatus* should be considered with appropriate biosafety and containment measures because of its opportunistic pathogenic nature, the thermotolerant physiology and enzymatic capacity of the isolates evaluated in this study make them valuable models for the development of robust lignocellulolytic SSF processes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomass6040054/s1>, Table S1. Cellulase and xylanase production by 75 thermophilic fungal strains cultivated under solid-state fermentation (SSF) at 45 °C for 48 h; Table S2. ANOVA results for cellulase maximum productivity using *A. fumigatus* 5S2 strain (2³ factorial design); Table S3. ANOVA results for xylanase maximum productivity using *A. fumigatus* 5S2 strain (2³ factorial design); Table S4. ANOVA results for cellulase maximum productivity using *Aspergillus* sp. 1.6C strain (2³ factorial design); Table S5. ANOVA results for xylanase maximum productivity using *Aspergillus* sp. 1.6C strain (2³ factorial design).

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