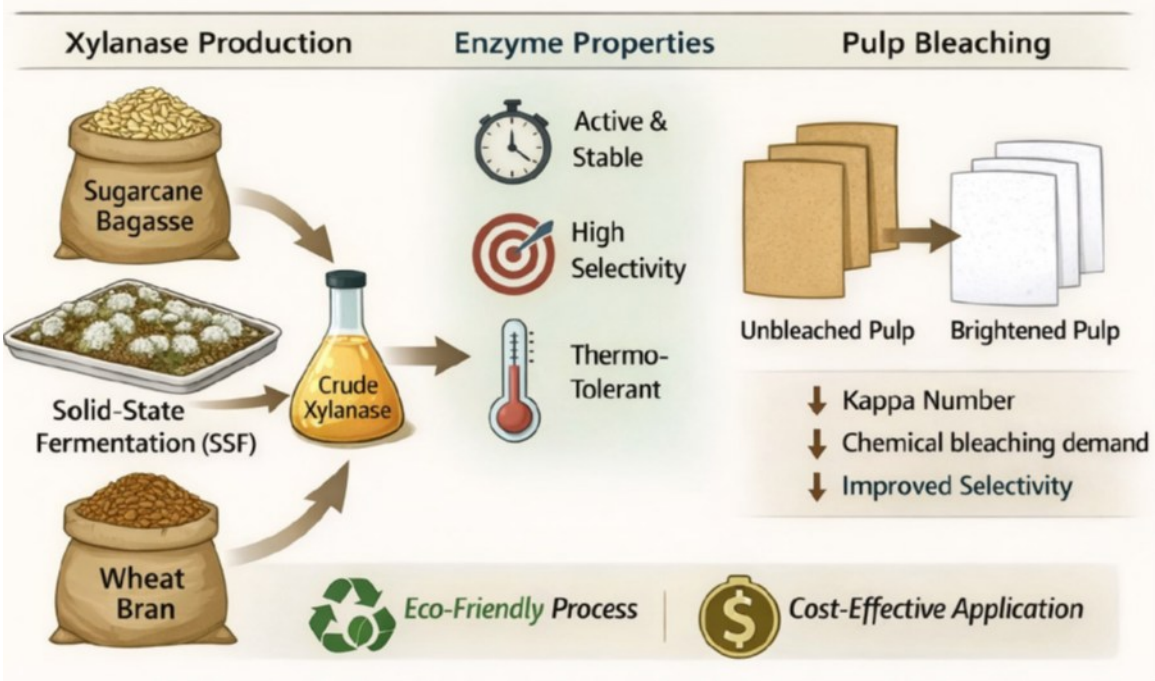
Agro-residue Valorization for Thermostable Xylanase Production by *Aspergillus caespitosus* and its Eco-friendly Application in Pulp Biobleaching

Diandra de Andrades [a], Valéria C. Sandrim [b] and Maria de Lourdes Teixeira de Moraes Polizeli [a,c,*]

** Corresponding author: polizeli@ffclrp.usp.br*

DOI: 10.15376/biores.21.1.1690-1705

GRAPHICAL ABSTRACT



Agro-residue Valorization for Thermostable Xylanase Production by *Aspergillus caespitosus* and its Eco-friendly Application in Pulp Biobleaching

Diandra de Andrades [a], Valéria C. Sandrim [b] and Maria de Lourdes Teixeira de Moraes Polizeli [a,c,*]

The fungus *Aspergillus caespitosus* was cultivated under solid-state fermentation (SSF) using wheat bran (WB) and sugarcane bagasse (SCB) as agro-industrial substrates to produce xylanase. WB supported the highest enzymatic activity (approximately 1100 U g^{-1} dry substrate), while pretreatment of SCB with NaOH (AT-SCB) enhanced productivity to about 1500 U g^{-1}, confirming the positive effect of lignocellulosic modification. The optimal moisture ratio (1:3 water: solid) yielded approximately 2400 U g^{-1}, and supplementation with 1% NH_4NO_3 and trace salts further increased xylanase synthesis by approximately 40%. The crude extract retained more than 80% of its activity after 72 h at 50 °C, indicating good thermal stability. In kraft pulp biobleaching, treatment with WB-derived xylanase (10 U g^{-1} pulp, 50 °C, 3 h) resulted in a 15% reduction in the kappa number and a 2.5 ISO-point increase in brightness, with no detectable cellulose degradation. These findings demonstrate that *A. caespitosus* efficiently produced a thermostable and selective xylanase under SSF, highlighting the potential of agro-residue valorization for developing environmentally friendly processes in the pulp and paper industry.

DOI: 10.15376/biores.21.1.1690-1705

Keywords: Pulp bleaching; Fungal biocatalysts; Lignocellulosic substrates; Wheat bran; Bagasse

*Contact information: a: Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14040-901, SP, Brazil; b: Department of Pharmacology - São Paulo State University, 18618-689 - Botucatu, SP, Brazil; c: Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, 14049-900 Ribeirão Preto, SP, Brazil; * Corresponding author: polizeli@ffclrp.usp.br*

INTRODUCTION

The increasing demand for sustainable biocatalysts capable of replacing harsh chemical treatments has intensified research on microbial enzymes (Basso and Serban 2019). Among these, xylanases (EC 3.2.1.8) have received considerable attention due to their ability to hydrolyze xylan, the major hemicellulosic component of plant cell walls. These enzymes are used in several industrial processes, including food, feed, and biofuel production, as well as in the pulp and paper industry, where they serve as environmentally friendly bleaching agents that reduce chlorine-based chemical consumption and effluent toxicity (Polizeli *et al.* 2005; Immerzeel and Fiskari 2023; Abena and Simachew 2024).

Importantly, hemicelluloses themselves are not toxic, and xylanase treatment does not generate toxic compounds directly. Instead, by selectively hydrolyzing surface-associated xylan, xylanases enhance lignin accessibility during subsequent chemical

bleaching stages, thereby reducing the demand for chlorine-based reagents. Consequently, the formation of toxic chlorinated organic compounds derived mainly from lignin is reduced, including adsorbable organic halides (AOX) and chlorophenols (Bajpai 2012).

There is a common perception among industrial stakeholders that enzymatic treatments may be relatively slow when compared with conventional chemical bleaching processes. The perception has contributed to a cautious approach toward their broader adoption. Factors such as reaction time, enzyme stability under alkaline and high-temperature conditions, mass transfer limitations in heterogeneous pulp systems, and integration into existing bleaching sequences are frequently cited as practical challenges. In addition, economic considerations related to enzyme dosage and overall process costs influence industrial decision-making. Consequently, current research efforts have focused on improving enzyme performance and thermostability, as well as on developing cost-effective production and application strategies suitable for industrially relevant conditions (Bajpai 2012; Immerzeel and Fiskari 2023).

Filamentous fungi are known to be efficient producers of extracellular enzymes, often displaying high activity and stability across a wide range of temperatures and pH (Dukare *et al.* 2023). However, commercial enzyme production remains limited by the high cost of substrates and process conditions, as downstream operations often constitute the largest share of bioprocess expenses and can reach up to 80% of total costs (Guajardo and Schrebler 2024). In biorefinery contexts, successful implementation further depends on developing enzyme cocktails that can be produced efficiently and at an affordable price (Pan *et al.* 2025). Solid-state fermentation (SSF) represents an advantageous alternative to submerged fermentation, as it enables the use of inexpensive agro-industrial residues, offers higher product concentration, and mimics the natural habitat of filamentous fungi, thus enhancing enzyme yields (Thomas *et al.* 2013).

Several lignocellulosic by-products, such as wheat bran (WB) and sugarcane bagasse (SCB), have been investigated as substrates for fungal enzyme production under SSF (Medeiros *et al.* 2022; Šekuljica *et al.* 2023). WB is rich in nutrients and exhibits a high water-retention capacity, whereas SCB, after pretreatment, provides an abundant and renewable carbon source (Hemery *et al.* 2010). The choice of substrate and the adjustment of culture parameters are therefore crucial to achieving high enzyme yields and ensuring stability during processing (Thomas *et al.* 2013; Soccol *et al.* 2017).

Beyond production, the application of fungal xylanases remains an active topic of study. In the pulp and paper industry, these enzymes participate in biobleaching by selectively hydrolyzing xylan–lignin linkages, facilitating lignin removal in subsequent alkaline extraction and improving pulp brightness without damaging cellulose (Abena and Simachew 2024). Some studies have addressed xylanase production under SSF or evaluated the application of xylanases in pulp biobleaching (Bajpai 2012). Reports integrating optimization of enzyme production with subsequent evaluation of biobleaching performance remain limited (Szendefy *et al.* 2003). Then, this work focused on optimizing xylanase synthesis by *A. caespitosus* using WB and SCB as substrates. A further emphasis was on evaluating the enzyme's biobleaching potential. The findings contribute to the development of sustainable enzymatic systems for the pulp and paper industry.

EXPERIMENTAL

All experiments were conducted using three independent biological replicates (n = 3). Enzyme activity assays were performed in duplicate technical replicates for each biological replicate. Results are expressed as mean ± standard deviation (SD).

Microorganism and Maintenance

Aspergillus caespitosus was isolated from soil and taxonomically identified by the Fundação André Tosello (Campinas, Brazil). Identification was performed based on macro- and microscopic morphological characteristics, using classical taxonomic keys for the genus *Aspergillus*. The strain was maintained on potato dextrose agar (PDA) plates and stored under laboratory conditions for subsequent experiments.

Substrates and Pretreatment

Wheat bran (WB) and sugarcane bagasse (SCB) were used as substrates for SSF. The substrates were washed, dried, milled, and sieved to obtain a uniform particle size (particle size of 1 to 2 mm). Alkali pretreatment of WB and BCA was performed as described. Briefly, 60 mL of 0.5 M NaOH was added to 10 g of WB or SCB, and the mixture was autoclaved at 121 °C for 20 min. After treatment, the substrates were washed with tap water until neutral pH and subsequently dried at 60 °C. The moisture content was maintained between 72 and 79%.

Solid-state Fermentation Conditions

For xylanase production, *A. caespitosus* was cultivated at 30 to 40 °C for 3 to 6 or 6 to 11 days in 95 mL Erlenmeyer flasks containing 2 g of either WB or SCB supplemented with 0 to 16 mL of tap or distilled water. This corresponded to an approximate solid loading of 0.125 to 0.25 g dry substrate mL^{-1} of added liquid (equivalent to 12.5 to 25 g dry substrate per 100 mL of liquid). In some experiments, Vogel's salts (Vogel 1964) or SR salts (Rizzatti *et al.* 2001) were added to the medium at 5% (v/v). Additional supplements tested included glucose, yeast extract, peptone, ammonium nitrate, or ammonium sulfate.

Enzyme Extraction

At the end of the incubation period, enzymes were extracted from the fermented solids by adding 30 to 180 mL of distilled water and shaking at 150 rpm and 4 °C for 30 min. The mixture was vacuum-filtered, and the mycelium was discarded unless multiple sequential extractions were being evaluated. The filtrates were dialyzed and used for xylanase and protease assays.

Enzyme Assays

Xylanase activity was determined according to Bailey *et al.* (1992) using birchwood xylan (Sigma, St. Louis, MO, USA) as substrate. The reaction mixture (0.5 mL) contained 0.25 mL of 1% (w/v) xylan, 0.15 mL of 100 mM MES buffer (pH 6.5), and 0.1 mL of enzyme extract. After incubation at 55 °C, the reducing sugars released were quantified using the dinitrosalicylic acid (DNS) method (Miller 1959), using xylose as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars per minute under the assay conditions.

Protease activity was assayed using casein as substrate. A mixture of 4 mL of 1% (w/v) casein in 0.1 M Tris–HCl buffer (pH 7.5) and 1 mL of enzyme extract was incubated at 40 °C. Aliquots (1 mL) were withdrawn and mixed with 1 mL of 10% (w/v) trichloroacetic acid. After incubation at 4 °C for 12 h, the samples were centrifuged at 3,000 rpm for 10 min, and the supernatant was used to quantify protease activity by the Lowry method (Lowry *et al.* 1951), using tyrosine as a standard. One unit of protease activity was defined as the amount of enzyme producing 1 μg of tyrosine per minute.

Pulp Bleaching

Unbleached kraft pulp derived from *Eucalyptus* spp. was kindly provided by Bahia Sul Co. (Brazil). Enzyme dosages were applied at 10 U per g of oven-dried pulp. The pulp consistency was adjusted to 10% (w/v), and the pH was adjusted to 6.0 to 6.5 with sulfuric acid. Control treatments were performed in the absence of enzymes, using distilled water under conditions identical to those used for enzyme treatments.

For bleaching assays, 10 g of dry pulp was mixed with the enzyme solution in sealed plastic bags and incubated at 55 °C for 120 min. After incubation, pulps were washed with 350 mL of distilled water. Subsequently, 5 g of each treated pulp was incubated in 2.2% NaOH at 65 °C for 60 min (12% consistency), followed by washing and drying at 60 °C overnight. The release of chromophores was evaluated by measuring absorbance at 237, 270, and 435 nm (Patel *et al.* 1993). Absorbance at 237 nm is associated with the presence of low-molecular-weight aromatic compounds released from lignin. Absorbance at 270 nm reflects aromatic and conjugated lignin-derived compounds remaining in the pulp. Measurements at 435 nm are commonly used as indicators of pulp color and chromophore content and are directly related to brightness changes during bleaching processes. The kappa number was determined according to TAPPI method T-236 os-76, defined as the volume (mL) of 0.1 N $KMnO_4$ consumed by 0.5 to 1.0 g of oven-dried pulp. Pulp viscosity was determined by dissolving samples in cupriethylenediamine and measuring the viscosity of 0.5% solutions at 25 °C using a capillary viscometer.

RESULTS AND DISCUSSION

Substrates

Agro-residues are attractive carbon sources because they are abundant and inexpensive, and they simultaneously reduce environmental impact.

Thus, an initial screening of different agro-industrial residues was conducted to identify suitable substrates for xylanase production under SSF (Fig. 1), including cassava flour (CF), rice straw (RS), oat, wheat bran (WB), and sugarcane bagasse (SCB). The highest enzyme activities were observed with WB and SCB, which yielded approximately 90% higher xylanase levels than the other substrates tested. These findings are consistent with recent reports highlighting WB as a cost-effective and nutrient-rich substrate for fungal xylanase production in SSF (Franco *et al.* 2023; Nunes *et al.* 2025). Other lignocellulosic residues, especially SCB, have been successfully used as substrates in SSF for xylanase production, particularly when combined with physicochemical pretreatments to improve accessibility (Alokika and Singh 2020). Visual inspection of fungal growth suggested that *A. caespitosus* developed more on WB, because this substrate provides a richer nutrient supply (Abena and Simachew 2024). Additionally, WB cultures are

associated with increased proteolytic activity, which can negatively affect enzyme stability during extraction and storage (Ferreira *et al.* 1999).

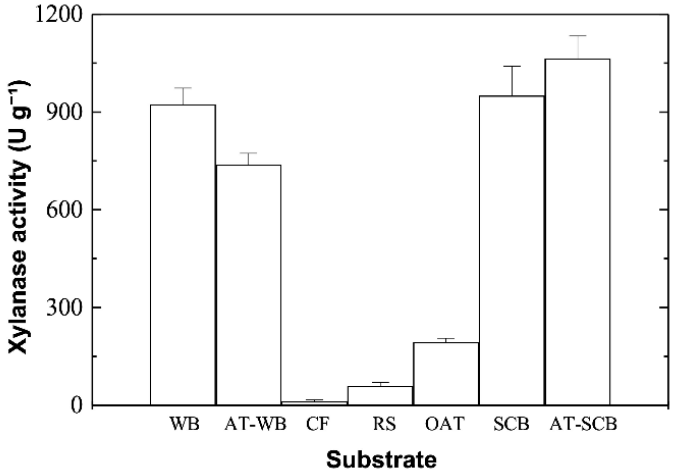


Fig. 1. Xylanase production by *A. caespitosus* cultivated under SSF using different agro-residues at 30 °C for 11 days for all treatments, using a fixed incubation time to enable comparative evaluation of production trends among substrates and supplementation conditions. WB (wheat bran), AT-WB (wheat bran with alkaline treatment), CF (cassava flour), RS (rice straw), OAT (oat flakes), SCB (sugarcane bagasse), and AT-SCB (sugarcane bagasse with alkaline treatment). The data reflect an initial substrate-screening experiment conducted under identical conditions.

The effect of alkaline pretreatment (AT) of WB and SCB on xylanase production was also assessed (Fig. 1). Pretreatment of WB reduced xylanase activity by 20%, suggesting that essential nutrients required for fungal development and enzyme synthesis were removed during the process. Similar nutrient losses have been reported in studies on alkali-extracted lignocellulosic substrates (Puițel *et al.* 2022). In contrast, AT-SCB cultures exhibited enhanced enzyme production. This improvement is likely due to the higher lignocellulosic content and structural rigidity of SCB, where NaOH pretreatment disrupts fibers and partially removes lignin, thereby increasing xylan accessibility to the enzyme (Yuan *et al.* 2021). Similar observations have been reported in a recent review, which emphasizes the effectiveness of alkaline pretreatment in improving the enzymatic digestibility of lignocellulosic biomass (Kululo *et al.* 2025).

Effect of Substrate–Water Ratio

Moisture content critically influences SSF performance: values above the optimum may reduce substrate porosity and impair oxygen diffusion, whereas values below the optimum decrease substrate swelling and nutrient solubilization, thereby limiting fungal metabolism (Wang *et al.* 2023). In many enzyme fermentations, increasing moisture increases activity up to a threshold, beyond which excessive moisture causes declines (Darabzadeh *et al.* 2019).

In WB cultures (Fig. 2A), xylanase activity at low water addition (0 to 1.5 mL) was about 64% lower than in cultures with 2 mL of water added. At water levels ≥ 2 mL, proteolytic activity and pH rose (Fig. 2B), likely contributing to reduced xylanase output. Excess moisture may both promote protease activity and reduce efficient gas transfer in the matrix (Guillaume *et al.* 2019).

In SCB cultures, the optimum was at 4 mL water addition (Fig. 2C). Proteolytic activity in those cultures was approximately fivefold lower than in WB (Fig. 2D),

indicating minimal protease interference. The comparatively stable balance between xylanase and protease activity across water levels suggests that SCB supports more consistent enzyme yields under varying moisture conditions, in agreement with recent reports confirming the suitability of SCB as an efficient substrate for xylanase production in SSF (Alokika and Singh 2020).

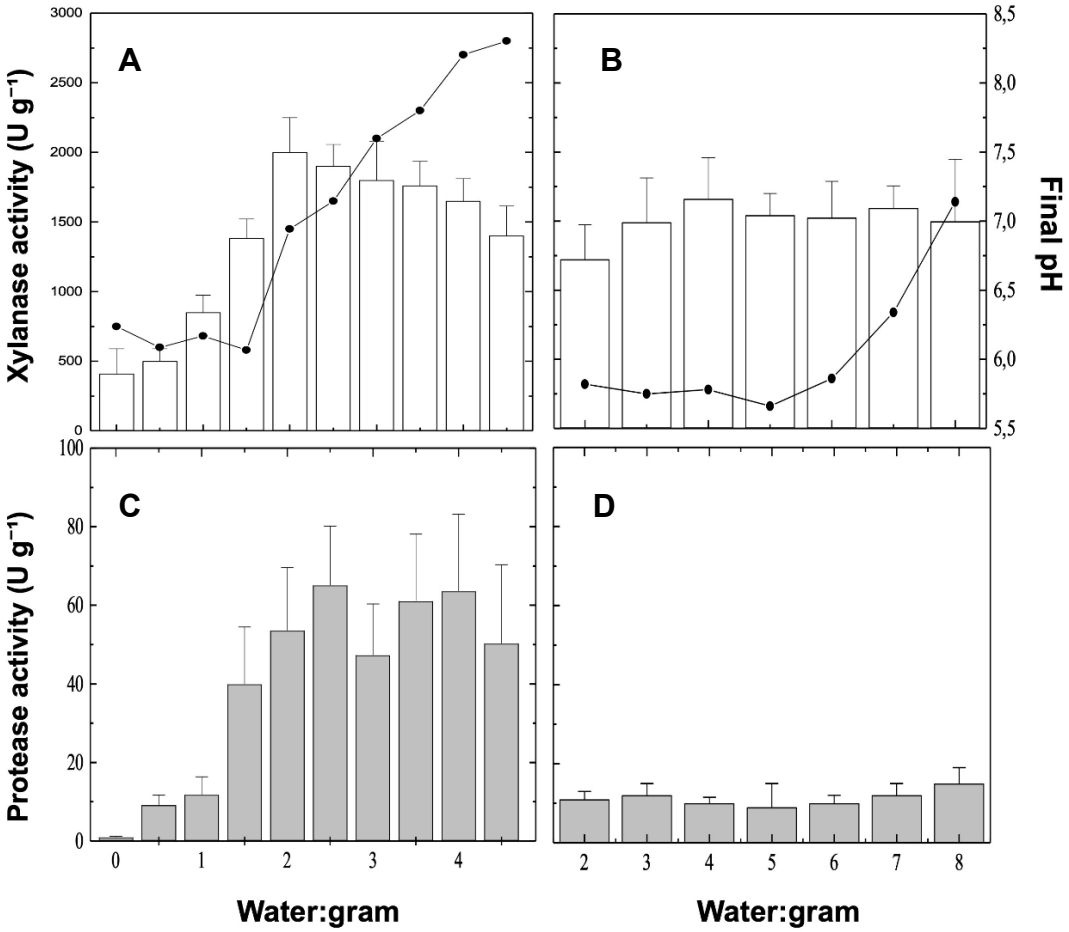


Fig. 2. Effect of initial moisture ratio on xylanase (☐) and protease (■) activities of *A. caespitosus* grown under SSF with WB (A, C) or SCB (B, D) at 30 °C for 5 and 11 days, respectively. WB (wheat bran) and SCB (sugarcane bagasse). Panels A and C share the same horizontal axis, as do panels B and D. Panels A and B, and C and D, share a common vertical axis and scale.

Time and Temperature of Culture

The influence of incubation time and temperature on xylanase production was evaluated at 30 and 40 °C using WB and SCB as substrates (Fig. 3A). Maximum enzyme activity was observed at 30 °C after 3 days in WB cultures and 6 days in SCB cultures. Under these conditions, xylanase yields were 31.2% and 69.7% higher, respectively, than those in cultures incubated at 40 °C for the same periods.

Proteolytic activity was generally lower at 40 °C for both substrates (Fig. 3B). This suggests that, although elevated temperatures may suppress protease production, they also impair fungal metabolism and enzyme synthesis, leading to reduced xylanase yields. These results are consistent with previous reports showing that moderate temperatures (25 to 35 °C) favor fungal growth and enzyme secretion in SSF. In contrast, higher temperatures often reduce enzyme output due to thermal stress and altered substrate utilization (Abena and Simachew 2024).

Wheat bran is known for its high water absorption and retention capacity. This can be mainly attributed to its fiber and arabinoxylan content, as well as its richer nutrient composition compared with other agro-residues (Hemery *et al.* 2007). These properties reduce moisture loss by evaporation during SSF and contribute to more stable fungal metabolism under relatively humid conditions. Such characteristics explain its frequent use as a preferred substrate in SSF and are further supported by studies linking wheat bran's water absorption and swelling properties with enhanced enzyme production (El-Shishtawy *et al.* 2014).

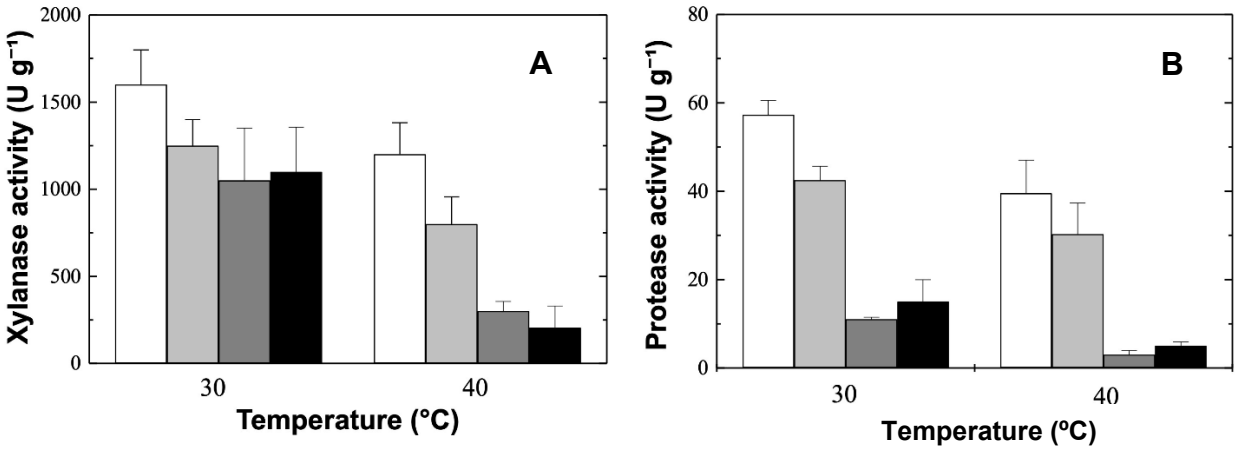


Fig. 3. Influence of incubation temperature (30 °C and 40 °C) and cultivation time on xylanase (A) and protease (B) activities during SSF. Fungus was cultivated on wheat bran (WB), by 3 (□), and 6 (■) days, or on sugarcane bagasse (SCB) by 6 (■), and 11 (■) days.

Effect of Medium Supplementation on Xylanase Production

Supplementation with different nitrogen sources significantly influenced xylanase production (Tables 1 and 2). In WB cultures, low nitrogen concentrations (0.5 to 1.0%) improved enzyme activity, whereas higher nitrogen supplementation (5.0%) decreased yields. The highest production was obtained with 0.5% ammonium nitrate, followed by 0.5% peptone and 1.0% yeast extract, which enhanced xylanase activity by 28, 25, and 22%, respectively, compared with the control.

Table 1. Effect of Nitrogen Sources on Xylanase Production by *A. caespitosus* under Solid-state Fermentation Using Wheat Bran (WB) as Substrate

Nitrogen Sources	Concentration (w/w)	Filtrate pH Final	Xylanase Activity (U g^{-1})
Yeast extract	0.5	6.92	1740 ± 55
	1.0	6.61	1954 ± 59
	5.0	6.20	1310 ± 38
Ammonium nitrate	0.5	6.30	2040 ± 73
	1.0	6.72	1802 ± 61
	5.0	5.90	682 ± 11
Peptone	0.5	6.61	1996 ± 69
	1.0	6.50	1722 ± 50
	5.0	6.10	1705 ± 47
Ammonium sulphate	0.5	6.80	1771 ± 66
	1.0	6.80	1781 ± 52
	5.0	5.20	1544 ± 44
Control	-	6.72	1600 ± 50

A. caespitosus was cultivated in SSF supplemented with 2 g of WB plus 4 mL of tap water for 5 days at 30 ºC. The volume of distilled water employed for enzyme extraction was 180 mL.

Table 2. Effect of Nitrogen Supplementation on Xylanase Activity of *A. caespitosus* during SSF, Using Sugarcane Bagasse (SCB) as Substrate

Nitrogen Sources	Concentration % (w/w)	Filtrate pH Final	Xylanase Activity (U g^{-1})
Yeast extract	10.0	6.96	1956 ± 60
Peptone	10.0	6.90	2230 ± 69
Ammonium Sulphate	10.0	6.47	1056 ± 36
Control	-	6.21	983 ± 21

A. caespitosus was cultivated in SSF supplemented with 2 g of SCB plus 8 mL of tap water for 11 days at 30 ºC. The volume of distilled water employed for enzyme extraction was 100 mL.

In SCB cultures, supplementation with 10% yeast extract, peptone, or ammonium sulfate was evaluated to compensate for the lower nutritional content of this residue. Maximum xylanase activity was detected in peptone-supplemented cultures, yielding 2.3-fold higher activity than the control (Table 2). Further optimization revealed that 6% peptone was the most effective concentration (Table 3), confirming the nutritional limitations of SCB and highlighting the beneficial role of peptone as both a nitrogen and a carbon source for fungal growth and enzyme production. Peptone concentrations are expressed on a dry substrate basis (w/w). Similar findings have been reported in other SSF systems, where the type and concentration of nitrogen source markedly influenced xylanase yields, with organic sources such as peptone frequently outperforming inorganic salts (Thomas *et al.* 2013).

These findings indicate that WB requires complementary nitrogen sources for optimal enzyme synthesis, as previously observed in SSF studies where nitrogen supplementation enhanced xylanase yields (Lemos *et al.* 2001). The superior performance of ammonium nitrate may be associated with its ability to repress protease activity, thereby improving xylanase stability, as also observed for microbial systems where ammonium salts reduced protease production (Sharma *et al.* 2017).

Table 3. Effect of Peptone Concentration on Xylanase Production under Solid-state Fermentation Using Sugarcane Bagasse (SCB)

Peptone Concentration (w/w)	Xylanase Activity (U g^{-1})
0.2	909 ± 26
3.0	1550 ± 56
6.0	2750 ± 88
12.0	2232 ± 67
20.0	2601 ± 82
30.0	2444 ± 63

A. caespitosus was cultivated in SSF with 2 g of SCB plus 8 mL of tap water for 11 days at 30 ºC. The volume of distilled water employed for enzyme extraction was 100 mL. Peptone concentration is expressed as a percentage (w/w) relative to the dry weight of the substrate.

From a process perspective, these results indicate that although higher peptone concentrations could maximize xylanase production in poor substrates, such as SCB, the resulting increase was not disproportionately greater than that achieved with other nitrogen sources at lower concentrations. Therefore, while elevated peptone levels may help overcome substrate limitations under laboratory conditions, moderate nitrogen levels may represent a more favorable balance between enzyme yield and process cost, particularly for large-scale solid-state fermentation applications (Pandey *et al.* 2003).

The effect of mineral supplementation was also evaluated by adding Vogel and SR salt solutions to the cultures (Fig. 4). In WB cultures, neither salt solution improved xylanase activity; in fact, tap water supported the highest enzyme levels. By contrast, in SCB cultures, Vogel salts enhanced enzyme production, whereas SR salts decreased yields. These results indicate that the mineral composition of the medium plays a critical role in fungal metabolism and xylanase synthesis under SSF (Pandey 2003; Soccol *et al.* 2017).

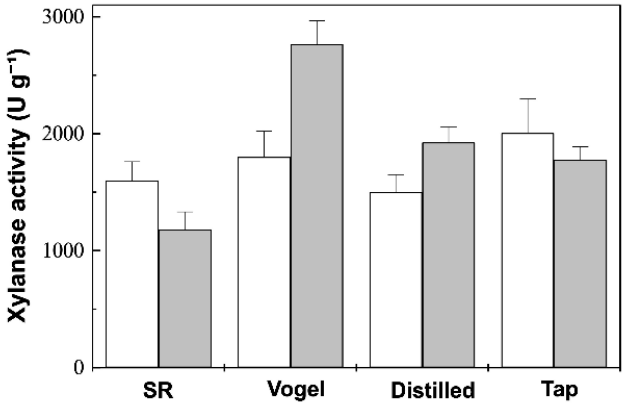


Fig. 4. Effect of mineral salt supplementation on xylanase activity in crude extracts obtained from SSF with wheat bran (WB) (□) or sugarcane bagasse (SCB) (■) at 30 ºC for 5 and 6 days, respectively.

The effect of glucose supplementation (2 to 10% w/w) was also investigated in WB and SCB cultures (Fig. 5). Unlike what is commonly observed in submerged fermentation, where glucose frequently triggers catabolite repression, no inhibitory effect was detected in SSF. Instead, enzyme production increased by 30% and 24% in WB and SCB cultures, respectively, compared with the controls without glucose addition. This observation is consistent with previous reports indicating that, under SSF, the limited diffusion of nutrients and lower water activity reduce the likelihood of repression by simple sugars (Polizeli *et al.* 2005; Thomas *et al.* 2013).

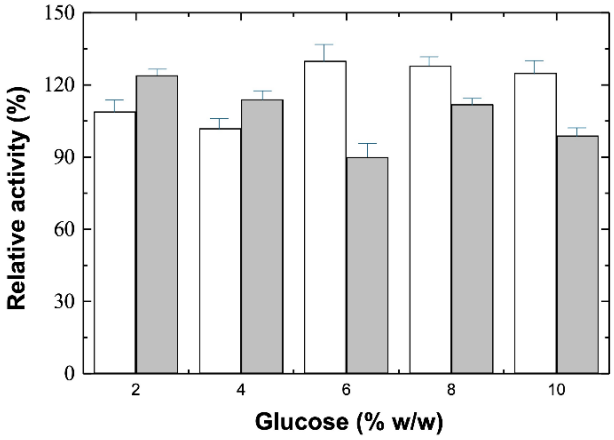


Fig. 5. Effect of glucose supplementation on xylanase production by *A. caespitosus* under SSF using wheat bran (WB) (☐) or sugarcane bagasse (SCB) (■) at 30 ºC for 5 and 6 days, respectively.

Overall, these findings highlight the importance of medium supplementation in modulating xylanase synthesis under SSF. In addition to the type of nitrogen source, the balance between carbon and nitrogen is a critical determinant of enzyme yields, as reported in studies showing that C: N ratios strongly influence fungal metabolism and extracellular enzyme secretion (Pandey 2003; Thomas *et al.* 2013). The superior performance of organic nitrogen sources, such as peptone, in SCB cultures may be attributed to their dual roles as nitrogen donors and auxiliary carbon sources, as well as their provision of growth factors. Mineral ions also play regulatory roles; elements such as Mg^{2+} and Ca^{2+} often stimulate fungal growth and enzyme secretion, whereas excess Fe^{3+} can be inhibitory (Soccol *et al.* 2017). Finally, the absence of catabolite repression by glucose in SSF can be explained not only by limited nutrient diffusion and lower water activity but also by the heterogeneous microenvironments of solid substrates, which reduce uniform regulatory responses compared with submerged systems (Polizeli *et al.* 2005).

Enzymatic Extraction

Because enzymes can remain adsorbed to solid substrates even after initial recovery, the amount of xylanase retained in WB and SCB was evaluated after three successive extractions with different volumes of water (30, 60, and 180 mL) (Fig. 6).

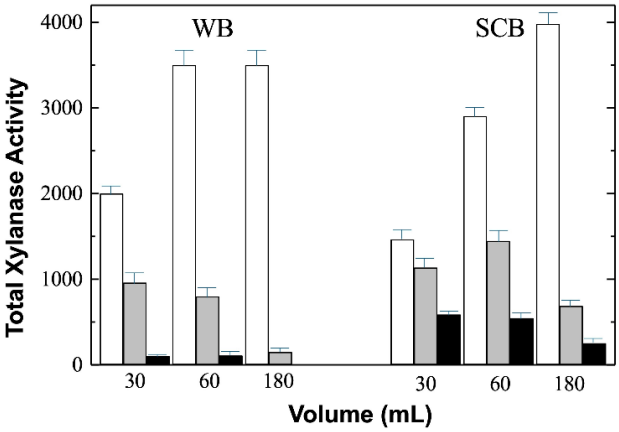


Fig. 6. Distribution of xylanase activity recovered from wheat bran (WB) and sugarcane bagasse (SCB) after extractions with 30, 60, and 180 mL of distilled water—first (☐), second (■), and third (■) extraction.

After the third extraction, essentially 100% of the xylanase activity was recovered. In both cultures, more than 50% of the total enzyme activity was released during the first extraction, except when only 30 mL of water was used. These results suggest that, to minimize processing costs in SSF, it is advantageous to perform at least two successive extractions to improve overall enzyme recovery. These findings are consistent with previous reports showing that, in SSF, a significant fraction of enzymes remains bound to the lignocellulosic matrix, making multiple washings or optimized extraction conditions necessary for efficient desorption. From an industrial perspective, optimizing product recovery is a critical step to reduce downstream costs and improve the economic feasibility of SSF (Soccol *et al.* 2017; Dukare *et al.* 2023; Immerzeel and Fiskari 2023).

Pulp Bleaching

To evaluate the bleaching potential of xylanases produced by SSF with WB or SCB, three parameters were assessed: UV absorbance at 237 to 270 nm, visible absorbance at 435 nm, and kappa number determination (Table 4). Both enzyme preparations, extracted from WB or SCB cultures, reduced the kappa number, particularly after alkaline extraction. The most pronounced effect was observed with xylanases from WB cultures, which may be attributed to their greater thermostability.

Table 4. Effect of Crude Xylanase Extracts from Solid-State Fermentation on the Biobleaching of Kraft Pulp

Parameters		SSF	
	Control	BCA	FT
Kappa number	11.9 (12.3)[a]	12.2 (11.7)	11.8 (11.4)
Abs 237	0.0665	0.0665	0.0665
Abs 270	0.4144	0.5361	0.5782
Abs 435	0.0356	0.0539	0.0563
Viscosity (cp)	35.9	37.5	39.5
Kappa number reduction (%)	-	0 (4.87)	0.84 (7.32)

[a]Kappa number determined after alkaline extraction. Each assay was performed with 10 g of pulp treated with 100 U of enzyme at 55 °C and pH 6.0 to 6.5 for 2 h. Values are mean ± SD (n = 3).

At 237 nm, absorbance values were similar among the three pulps analyzed. In contrast, increases at 270 and 435 nm relative to the control pulp indicated effective lignin solubilization and chromophore release, consistent with the observed reduction in kappa number.

The mechanism underlying xylanase-assisted pulp bleaching involves the hydrolysis of xylan chains linked to lignin–cellulose complexes (Lawoko *et al.* 2005), thereby increasing lignin accessibility during subsequent alkaline extraction rather than promoting direct cellulose fiber degradation. Partial hydrolysis of surface-associated xylan may disrupt this interfacial network, facilitating lignin mobilization and removal. Notably, the increase in pulp viscosity observed after enzymatic treatment indicates that cellulose integrity was preserved, supporting the selective action of xylanase on hemicellulose-associated structures. The superior performance of enzymes derived from WB cultures may also be related to their higher thermostability, a characteristic considered essential for biobleaching processes conducted under alkaline, elevated-temperature industrial conditions (Lawoko *et al.* 2005).

From an environmental perspective, incorporating xylanases into bleaching sequences enables partial substitution for chlorine-based reagents, thereby reducing effluent toxicity and ecological impact. Recent reviews confirm that fungal and bacterial xylanases can consistently reduce kappa number by 10 to 20% and enhance pulp brightness without compromising cellulose quality, supporting their integration into industrial-scale processes (Dukare *et al.* 2023; Immerzeel and Fiskari 2023).

From a process engineering perspective, the industrial implementation of enzymatic bleaching stages often raises concerns about residence time and the potential need for large, agitated chests, which may entail a significant capital investment. The required volume of such units is directly related to the kinetics of enzyme action, whereas enzyme dosage and contact time are interdependent. In practice, higher enzyme dosages can shorten treatment times and reduce required reactor volumes, whereas longer residence times may allow operation at lower enzyme loadings, potentially decreasing operating costs. Although the present study was not designed to optimize reaction kinetics at the industrial scale, the observed bleaching effects obtained under moderate incubation times and enzyme dosages suggest that xylanase-assisted bleaching can be compatible with existing process configurations when appropriately integrated. These considerations highlight the importance of balancing enzyme dosage and residence time in future scale-up studies to minimize both capital and operating costs.

CONCLUSIONS

1. This study demonstrated that *Aspergillus caespitosus* was able to efficiently produce xylanase under solid-state fermentation (SSF) using low-cost agro-industrial residues such as wheat bran (WB) and sugar cane bagasse (SCB). Among the evaluated conditions, WB proved to be the most effective substrate, yielding higher enzymatic levels due to its richer nutrient composition and superior water retention capacity, both of which favor fungal metabolism. Supplementation with nitrogen sources and mineral salts further modulated enzyme synthesis, highlighting the importance of nutrient balance in optimizing SSF performance.
2. The produced enzyme exhibited suitable stability and catalytic performance for industrial application, as evidenced by its effectiveness in pulp biobleaching. Treatment of kraft pulp with the WB-derived xylanase preparation resulted in significant lignin removal, as indicated by kappa number reduction and increased absorbance at 270 and 435 nm, while maintaining cellulose integrity. These results confirm the potential of *A. caespitosus* xylanase as a selective, eco-friendly biocatalyst for the partial replacement of chemical reagents in bleaching processes.
3. Overall, the findings reinforce the value of SSF as a sustainable platform for enzyme production from renewable substrates. Future work should focus on process scale-up and downstream optimization to enable the industrial application of thermostable fungal xylanases in pulp and paper manufacturing.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the São Paulo Research Foundation (FAPESP) (2014/50884-5, 2018/07522-6, and 2023/01547-5) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (465319/2014-9) for financial support. Research scholarships were granted to DA by FAPESP (2020/15510-8) and to MLTMP by CNPq Productivity Scholarships (310340/2021-7). Mauricio de Oliveira provided technical support.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

REFERENCES

Abena, T., and Simachew, A. (2024). "A review on xylanase sources, classification, mode of action, fermentation processes, and applications as a promising biocatalyst," *BioTechnologia* 105(3), 273-285. https://doi.org/10.5114/bta.2024.141806

Alokika, and Singh, B. (2020). "Enhanced production of bacterial xylanase and its utility in saccharification of sugarcane bagasse," *Bioprocess and Biosystems Engineering* 43(6), 1081-1091. https://doi.org/10.1007/s00449-020-02306-8

Bailey, M. J., Biely, P., and Poutanen, K. (1992). "Interlaboratory testing of methods for assay of xylanase activity," *Journal of Biotechnology* 23(3), 257-270. DOI: https://doi.org/10.1016/0168-1656(92)90074-J

Bajpai, P. (2012). *Biotechnology for Pulp and Paper Processing*, Springer US, Boston, MA. DOI: 10.1007/978-1-4614-1409-4

Basso, A., and Serban, S. (2019). "Industrial applications of immobilized enzymes—A review," *Molecular Catalysis* 479, article 110607. https://doi.org/10.1016/j.mcat.2019.110607

Darabzadeh, N., Hamidi-Esfahani, Z., and Hejazi, P. (2019). "Optimization of cellulase production under solid-state fermentation by a new mutant strain of *Trichoderma reesei*," *Food Science & Nutrition* 7(2), 572-578. https://doi.org/10.1002/fsn3.852

Dukare, A., Sharma, K., Kautkar, S., Dhakane-Lad, J., Yadav, R., Nadanathangam, V., and Saxena, S. (2023). "Microbial xylanase aided biobleaching effect on multiple components of lignocelluloses biomass based pulp and paper: A review," *Nordic Pulp & Paper Research Journal* 38(3), 459-480. https://doi.org/10.1515/npprj-2023-0005

El-Shishtawy, R. M., Mohamed, S. A., Asiri, A. M., Gomaa, A. M., Ibrahim, I. H., and Al-Talhi, H. A. (2014). "Solid fermentation of wheat bran for hydrolytic enzymes production and saccharification content by a local isolate *Bacillus megatherium*," *BMC Biotechnology* 14(1), article 29. https://doi.org/10.1186/1472-6750-14-29

Ferreira, G., Boer, C. G., and Peralta, R. M. (1999). "Production of xylanolytic enzymes by *Aspergillus tamarii* in solid state fermentation," *FEMS Microbiology Letters* 173(2), 335-339. https://doi.org/10.1111/j.1574-6968.1999.tb13522.x

Franco, D. G., de Almeida, A. P., Galeano, R. M. S., Vargas, I. P., Masui, D. C., Giannesi, G. C., Ruller, R., and Zanoelo, F. F. (2023). "Exploring the potential of a new thermotolerant xylanase from *Rasamsonia composticola* (XylRc): Production using agro-residues, biochemical studies, and application to sugarcane bagasse saccharification," *3 Biotech* 14(1), article 3. https://doi.org/10.1007/s13205-023-03844-0

Guajardo, N., and Schrebler, R. A. (2024). "Upstream and downstream bioprocessing in enzyme technology," *Pharmaceutics* 16(1). https://doi.org/10.3390/pharmaceutics16010038

Guillaume, A., Thorigné, A., Carré, Y., Vinh, J., and Levavasseur, L. (2019). "Contribution of proteases and cellulases produced by solid-state fermentation to the improvement of corn ethanol production," *Bioresources and Bioprocessing* 6(1), 7. https://doi.org/10.1186/s40643-019-0241-0

Hemery, Y. M., Anson, N. M., Havenaar, R., Haenen, G. R. M. M., Noort, M. W. J., and Rouau, X. (2010). "Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions," *Food Research International* 43(5), 1429-1438. https://doi.org/10.1016/j.foodres.2010.04.013

Hemery, Y., Rouau, X., Lullien-Pellerin, V., Barron, C., and Abecassis, J. (2007). "Dry processes to develop wheat fractions and products with enhanced nutritional quality," *Journal of Cereal Science* 46(3), 327-347. https://doi.org/10.1016/j.jcs.2007.09.008

Immerzeel, P., and Fiskari, J. (2023). "Synergism of enzymes in chemical pulp bleaching from an industrial point of view: A critical review," *The Canadian Journal of Chemical Engineering* 101(1), 312-321. https://doi.org/10.1002/cjce.24374

Kululo, W. W., Habtu, N. G., Abera, M. K., Sendekie, Z. B., Fanta, S. W., and Yemata, T. A. (2025). "Advances in various pretreatment strategies of lignocellulosic substrates for the production of bioethanol: A comprehensive review," *Discover Applied Sciences* 7(5), article 476. https://doi.org/10.1007/s42452-025-06748-1

Lawoko, M., Henriksson, G., and Gellerstedt, G. (2005). "Structural differences between the lignin−carbohydrate complexes present in wood and in chemical pulps," *Biomacromolecules* 6(6), 3467-3473. DOI: https://doi.org/10.1021/bm058014q

Lemos, J. L. S., Fontes, M. C. de A., and Pereira, N. (2001). "Xylanase production by *Aspergillus awamori* in solid-state fermentation and influence of different nitrogen sources," *Applied Biochemistry and Biotechnology* 91(1), 681-689. https://doi.org/10.1385/ABAB:91-93:1-9:681

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). "Protein measurement with the Folin phenol reagent," *The Journal of Biological Chemistry* 193, 265-275.

Medeiros, T. D. M., Dufossé, L., and Bicas, J. L. (2022). "Lignocellulosic substrates as starting materials for the production of bioactive biopigments," *Food Chemistry: X* 13, article 100223. https://doi.org/10.1016/j.fochx.2022.100223

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry* 31(3), 426-428. https://doi.org/10.1021/ac60147a030

Nunes, J. S., de Araújo Padilha, C. E., Costa, I. O., da Silva, R. P., Alcântara, M. A., dos Santos, E. S., de Carvalho Gonçalves, L. C. T., de Araújo, D. A. M. (2025). "Production of xylanases from *Penicillium* sp. FSDE15 using agro-industrial residues and their biotechnological potential," *Waste and Biomass Valorization* 16(6), 2969-2983. https://doi.org/10.1007/s12649-024-02840-6

Pan, Y., Lu, Y., Dong, Y., Sun, Y., Tang, R., Xiao, Y., Gao, B., and Zhu, D. (2025). "The enzyme cocktail produced by endophytic *Chaetomium globosum* unlocks *in situ* production of sugars and shows great potential application in biorefinery," *Renewable Energy* 242, article 122449. https://doi.org/10.1016/j.renene.2025.122449

Pandey, A. (2003). "Solid-state fermentation," *Biochemical Engineering Journal* 13(2), 81-84. https://doi.org/10.1016/S1369-703X(02)00121-3

Patel, R. N., Grabski, A. C., and Jeffries, T. W. (1993). "Chromophore release from kraft pulp by purified *Streptomyces roseiscleroticus* xylanases," *Applied Microbiology and Biotechnology* 39(3), 405-412. https://doi.org/10.1007/BF00192102

Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A., and Amorim, D. S. (2005). "Xylanases from fungi: Properties and industrial applications," *Applied Microbiology and Biotechnology* 67(5), 577-591. https://doi.org/10.1007/s00253-005-1904-7

Puițel, A. C., Suditu, G. D., Danu, M., Ailiesei, G.-L., and Nechita, M. T. (2022). "An experimental study on the hot alkali extraction of xylan-based hemicelluloses from wheat straw and corn stalks and optimization methods," *Polymers* 14(9), article 1662. https://doi.org/10.3390/polym14091662

Rizzatti, A. C. S., Jorge, J. A., Terenzi, H. F., Rechia, C. G. V., and Polizeli, M. L. T. M. (2001). "Purification and properties of a thermostable extracellular β-D-xylosidase produced by a thermotolerant *Aspergillus phoenicis*," *Journal of Industrial Microbiology and Biotechnology* 26(3), 156-160. https://doi.org/10.1038/sj.jim.7000107

Šekuljica, N., Jakovetić Tanasković, S., Mijalković, J., Simović, M., Pavlović, N., Đorđević, N., Culetu, A., Gazikalović, I., Luković, N., Bakrač, J., and Knežević-Jugović, Z. (2023). "Xylanase production by solid-state fermentation for the extraction of xylooligosaccharides from soybean hulls," *Food Technology and Biotechnology* 61(4), 439-450. https://doi.org/10.17113/ftb.61.04.23.8073

Sharma, K. M., Kumar, R., Panwar, S., and Kumar, A. (2017). "Microbial alkaline proteases: Optimization of production parameters and their properties," *Journal of Genetic Engineering and Biotechnology* 15(1), 115-126. https://doi.org/10.1016/j.jgeb.2017.02.001

Soccol, C. R., da Costa, E. S. F., Letti, L. A. J., Karp, S. G., Woiciechowski, A. L., and de Souza Vandenberghe, L. P. (2017). "Recent developments and innovations in solid state fermentation," *Biotechnology Research and Innovation* 1(1), 52-71. https://doi.org/10.1016/j.biori.2017.01.002

Szendefy, J., Szakács, G., Kemény, S., and Christov, L. (2003). "*In situ* solid-state fermentation and utilization of xylanase in pulp bleaching," in: *Applications of Enzymes to Lignocellulosics*, ACS Symposium Series, American Chemical Society, pp. 255-284. DOI: https://pubs.acs.org/doi/abs/10.1021/bk-2003-0855.ch016

Thomas, L., Larroche, C., and Pandey, A. (2013). "Current developments in solid-state fermentation," *Biochemical Engineering Journal* 81, 146-161. https://doi.org/10.1016/j.bej.2013.10.013

Vogel, H. J. (1964). "Distribution of lysine pathways among fungi: Evolutionary implications," *The American Naturalist* 98(903), 435-446. https://doi.org/10.1086/282338

Wang, H., Sun, C., Yang, S., Ruan, Y., Lyu, L., Guo, X., Wu, X., and Chen, Y. (2023). "Exploring the impact of initial moisture content on microbial community and flavor generation in Xiaoqu baijiu fermentation," *Food Chemistry: X* 20, article 100981.

https://doi.org/10.1016/j.fochx.2023.100981
Yuan, Y., Jiang, B., Chen, H., Wu, W., Wu, S., Jin, Y., and Xiao, H. (2021). "Recent advances in understanding the effects of lignin structural characteristics on enzymatic hydrolysis," *Biotechnology for Biofuels* 14(1), article 205. https://doi.org/10.1186/s13068-021-02054-1

Article submitted: November 17, 2025; Peer review completed: December 8, 2025; Revised version received and accepted: December 23, 2025; Published: January 8, 2026.
DOI: 10.15376/biores.21.1.1690-1705