

Production and characterization of cellulases and hemicellulases from a consortium between *Pleurotus ostreatus* and *Aspergillus niger* cultured in agro-industrial wastes

Produção e caracterização de celulases e hemicelulases por um consórcio entre *Pleurotus ostreatus* e *Aspergillus niger* cultivados em resíduos agroindustriais

Producción y caracterización de celulases e hemicelulasas por un consórcio entre *Pleurotus ostreatus* y *Aspergillus niger* cultivados en residuos agroindustriales

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Abstract

The enzyme biosynthesis using agricultural wastes by solid state fermentation (SSF) and the study of their physicochemical properties are meaningful approaches to improve the biomass hydrolysis. Among them, β -glucosidases and β -xylosidases are key enzymes at the lignocellulose depolymerization, which act in the cleavage of oligosaccharides in monosaccharides. In this study, the production of hemicellulases and cellulases by *Pleurotus ostreatus* and *Aspergillus niger* monocultures or in consortium was investigated, using raw sugarcane bagasse (SB) and wheat bran (WB) as substrates. The highest enzymatic activities were observed in the crude extract produced by *P. ostreatus* PLO6 and *A. niger* SCBM4 consortium with 98.5, 62.9, 3.8, 12.4, 13.3 and 20.2 U/g for β -glucosidase (β -glu), β -xylosidase (Bxyl), filter paper cellulase (FPase), xylanase (Xyl), exoglucanase (Exgl) and endoglucanase (Engl), respectively. The pH and temperature effects on β -glu and β -xyl were characterized. Optimal activities were obtained at pH 4.0 and 45 °C for β -glu and 3.5 and 55 °C for β -xyl. Both enzymes were stable at acid pH and presented thermostability. The results indicated that the enzymatic cocktail demonstrated potential characteristics for future applications in saccharifications. The use of sugarcane bagasse and wheat bran for microbial growth contributed to aggregate value to these byproducts.

Keywords: Solid state fermentation; Ascomycete; Basidiomycete; (Hemi) Cellulolytic enzymes; Prospection.

Resumo

A biossíntese de enzimas utilizando resíduos agrícolas por fermentação em estado sólido (SSF) e o estudo de suas propriedades físico-químicas são abordagens significativas para melhorar a hidrólise da biomassa. Dentre elas, as β -glicosidases e β -xilosidases são enzimas-chave na despolimerização da lignocelulose, que atuam na clivagem de oligossacarídeos em monossacarídeos. Neste estudo, foi investigada a produção de hemicelulases e celulases por monoculturas de *Pleurotus ostreatus* e *Aspergillus niger* ou em consórcio, utilizando bagaço de cana-de-açúcar (SB) e farelo de trigo (WB) como substratos. As maiores atividades enzimáticas foram observadas no extrato bruto produzido pelo consórcio *P. ostreatus* PLO6 e *A. niger* SCBM4 com 98,5, 62,9, 3,8, 12,4, 13,3 e 20,2 U/g para β -glucosidase (β -glu), β -xilosidase (β -xyl), celulase de papel de filtro (FPase), xilanase (Xyl), exoglucanase (Exgl) e endoglucanase (Engl), respectivamente. Os efeitos do pH e da temperatura em β -glu e β -xyl foram caracterizados. As atividades ótimas foram obtidas em pH 4,0 e 45 °C para β -glu e 3,5 e 55 °C para β -xyl. Ambas as enzimas foram estáveis em pH ácido e apresentaram termoestabilidade. Os resultados indicaram que o coquetel enzimático demonstrou características potenciais para futuras aplicações em sacarificações. A utilização do bagaço da cana-de-açúcar e do farelo de trigo para o crescimento microbiano contribuiu para agregar valor a esses subprodutos.

Palavras-chave: Fermentação em estado sólido; Ascomiceto; Basidiomiceto; Enzimas (Hemi) celulolíticas; Prospecção.

Resumen

La biosíntesis de enzimas utilizando desechos agrícolas por fermentación en estado sólido (SSF) y el estudio de sus propiedades fisicoquímicas son enfoques significativos para mejorar la hidrólisis de la biomasa. Entre ellas, las β -glucosidasas y las β -xilosidasas son enzimas clave en la despolimerización de la lignocelulosa, que actúan en la escisión de oligosacáridos en monosacáridos. En este estudio se investigó la producción de hemicelulasas y celulasas por monocultivos de *Pleurotus ostreatus* y *Aspergillus niger* o en consorcio, utilizando como sustratos bagazo de caña de azúcar crudo (SB) y salvado de trigo (WB). Las mayores actividades enzimáticas se observaron en el extracto crudo producido por el consorcio *P. ostreatus* PLO6 y *A. niger* SCBM4 con 98.5, 62.9, 3.8, 12.4, 13.3 y 20.2 U / g para β -glucosidasa (β -glu), β -xilosidasa (β -xyl), papel de filtro celulasa (FPasa), xilanasa (Xyl), exoglucanasa (Exgl) y endoglucanasa (Engl), respectivamente. Se caracterizaron los efectos del pH y la temperatura sobre β -glu y β -xyl. Las actividades óptimas se obtuvieron a pH 4.0 y 45 °C para β -glu y 3.5 y 55 °C para β -xyl. Ambas enzimas fueron estables a pH ácido y presentaron termoestabilidad. Los resultados indicaron que el cóctel enzimático demostró características potenciales para futuras aplicaciones en sacarificaciones. El uso de bagazo de caña de azúcar y salvado de trigo para el crecimiento microbiano contribuyó al valor agregado de estos subproductos.

Palabras clave: Fermentación en estado sólido; Ascomiceto; Basidiomiceto; Enzimas (Hemi) celulolíticas; Prospeccción.

1. Introduction

Lignocellulosic wastes from agroindustry can be viable and low-cost raw materials of great potential for the production of enzymes, biofuels and biorefineries. Among biofuels, cellulosic ethanol can be synthesized from the depolymerization of lignocellulose in fermentable sugars (Toquero and Bolado, 2014). In Brazil, the ethanol obtained from sugarcane bagasse is a promising strategy, since this by-product is generated in large quantities and does not compete with the food chain (Lamounier et al. 2020). Hemicellulases and cellulases are lignocellulose-degrading enzymes responsible for the synergistic breakdown of biomass into free monosaccharides, with wide industrial application such as biofuels, pulp and paper industry, textile sector, detergents, feed, beverages, among others (Verma et al. 2020). The cellulose depolymerization occurs through the action of three kinds of cellulases: endoglucanases or endo-1,4- β -D-glucanases (EC 3.2.1.4), exoglucanases or exo- β -1,4-glucanases or cellobiohydrolases (EC 3.2.1.91) and β -1,4-glucosidases or cellobiases (EC 3.2.1.21). β -Glucosidases are key-enzymes in the cellulose bioconversion, which catalyze the hydrolysis of the glycosidic bond of cellobiose disaccharides in free glucose molecules (Rani et al. 2014). Among hemicellulases, xylanases or endo- β -1,4-xylanases (EC 3.2.1.8) and β -xylosidases or xylan β -1,4-xylosidases (3.2.1.37) are commonly employed for the xylan hydrolysis, the main polysaccharide in the hemicelluloses group, with a wide commercial application (Gomes et al., 2016).

Studies about the catalytic efficiency of enzymes from the (hemi)-cellulolytic complex have been developed in order to obtain high yields in fermentable monosaccharides (Canilha et al., 2012; Infanzón-Rodríguez et al. 2020). However, one of the chief limitations of this process is the use of high cost commercial enzymatic cocktails (Verma et al. 2020). On the other hand, the use of widely available agri-food residues, such as sugarcane bagasse, wheat bran and sugar beet, among others, for the production of enzymes by solid state fermentation (SSF), represents a worthwhile alternative to contribute in cost decrement (Rodrigues et al., 2020; Arruda et al., 2021). This way, SSF is considered a sustainable strategy since it allows the valorization of economical raw materials, increasing the viability of the process and minimizing the pollution caused by these environmental passives. Briefly, SSF simulates the natural habitat of microorganisms, allowing them to grow on a porous solid substrate with sufficient moisture to assure their metabolism and growth (Rodrigues et al., 2020). Fungal strains are preferred to develop in these substrates and produce different kinds of hydrolytic enzymes by SSF, providing useful destination for these materials and aggregating value to them (Rodrigues et al., 2017). Among them, *Aspergillus* species are known as excellent producers of cellulases, being *A. niger* and *A. fumigatus* recognized as good producers of β -glucosidases (Dos Santos et al. 2015; Dias et al. 2017). Wood and plant waste decomposers macrofungi, such as the species from the genus *Pleurotus* have

also been reported by their enzymatic complex capable to degradate lignocellulosic materials, being species from the genus *Pleurotus* very promising to be cultured in SSF due to its capacity to use lignin, hemicellulose and cellulose as sources of carbon (Da Luz et al., 2012; Araújo et al., 2021).

With the aim to improve the efficacy of hydrolytic processes, co-cultures between fungal species can be employed to enhance the biosynthesis of enzymes. For example, Rodrigues et al. (2020) evaluated the efficiency of fungal consortia of different species for the enzyme production by SSF and observed that the co-cultures containing *A. niger* among the inocula stood out for cellulases and hemicellulases production in comparison to monocultures. In this context, the present study evaluated the production of enzymes by the cultivation of the strains *Pleurotus ostreatus* PLO6 and *Aspergillus niger* SCBM4, in monocultures and in consortium, by SSF with sugarcane bagasse (SB) and wheat bran (WB) as substrates. The biosynthesis of cellulases (exoglucanase, endoglucanase, β -glucosidase and filter paper cellulase) and hemicellulases (xylanase and β -xylosidase) was investigated. Afterwards, the β -glucosidase and β -xylosidase present in the crude enzymatic extract from the fungal co-culture was characterized, in order to determine their optimal activities and stabilities against a wide range of pH and temperature, aiming future applications.

2. Methodology

2.1 Microorganisms

The *P. ostreatus* PLO6 strain was obtained from commercial shimeji and kindly donated by Dr. Maria Catarina Megumi Kasuya (Department of Microbiology, Federal University of Viçosa, Brazil). The *A. niger* SCBM4 strain was obtained from the Collection of Microorganisms of the Laboratory of Environmental Microbiology (Federal University of Uberlândia, Brazil). This strain was previously isolated from piles of raw sugar cane bagasse (Dos Santos et al. 2015). Both fungal strains were preserved in Petri dishes containing potato, dextrose agar medium (PDA, Sigma-Aldrich) at room temperature.

2.2 Substrates

Sugarcane bagasse (SB) and wheat bran (WB) were used as carbon sources in SSF for microbial growth. SB was kindly provided by Usina Vale do Tijuco (Uberaba, MG, Brazil) and WB sample was purchased from a local cereal market (Uberlândia, MG, Brazil). Samples of SB and WB were chemically characterized according to Technical Association of the Pulp and Paper Industry (TAPPI) and National Renewable Energy Laboratory (NREL) standard methods for contents of: moisture (TAPPI T257 om-85), ash (TAPPI T211 om-93), total Klason lignin (TAPPI 222 om-88) and cellulose and hemicelluloses (NREL LAP-002). The lignin, cellulose and hemicellulose percentages were also determined by high-performance liquid chromatography (HPLC, SHIMADZU) (Dias et al., 2017). Both substrates were washed with distilled water, dried at room temperature for 48 h. Later, they were milled in a crusher and sieved to around 0.6-1.0 cm particle size.

2.3 Solid State Fermentation

The SSF were performed in 250 mL Erlenmeyer flasks containing 2.5 g of SB and 2.5 g of WB, in a total of 5.0 g (1:1 w/w) of dry substrate, supplemented with 5 mL of sterile nutrient solution (0.5 g/L of ammonium sulfate - $(\text{NH}_4)_2\text{SO}_4$, 3.0 g/L of potassium phosphate monobasic - KH_2PO_4 , 0.5 g/L of magnesium sulfate heptahydrate - $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 g/L of calcium chloride - CaCl_2) and sterilized in autoclave at 121 °C for 20 min.

Fungal strains were individually inoculated in PDA medium at 28 °C for seven days or until the fungi cover the entire Petri dish. Afterwards, mycelial discs of 0.5 cm² of diameter from each preculture were removed and homogenized in 5 mL of nutrient solution, and then, inoculated in sterile flasks containing the substrates (Rodrigues et al., 2020). A total of ten discs

were inoculated for monocultures and five discs of each strain for the growth in consortium and the volume was completed with 10 mL of nutrient solution (65% moisture). SSF were carried out in three conditions: Fermentation 1 (*P. ostreatus* PLO6 monoculture), Fermentation 2 (*P. ostreatus* and *A. niger* consortium) and Fermentation 3 (*A. niger* SCBM4 monoculture).

Erlenmeyer flasks were incubated in duplicate at 28 °C for 14 days. At intervals of 24 h, two flasks were removed from the incubator, solubilized in 50 mL of distilled water, homogenized and subjected to orbital agitation for 30 min at 200 rpm for the extraction of the produced enzymes. The samples were filtered through nylon cloth and centrifuged at 5000 rpm for 1 h (Rodrigues et al., 2020). The supernatants containing the crude enzymatic extracts were aliquoted and stored at -20 °C for further assays. This process was repeated until the end of the SSF.

2.4 Enzymatic Activities

The quantifications of the enzymatic activities in the crude extracts were carried out in triplicates using substrates and reagents purchased from Sigma-Aldrich. The activity measurements were performed by spectrophotometry using an UV-Vis Spectrophotometer (METASH UV-5100).

2.5 β -Glucosidase and β -xylosidase

The activity of β -glucosidase was quantified using *p*-nitrophenyl- β -D-glucoopyranoside (PNPG, 4 mmol/L) as substrate in sodium citrate buffer solution (0.05 mol/L, pH 4.8), for 10 min at 40 °C. The amount of *p*-nitrophenol (*p*NP) released was quantified at 410 nm in an UV-Vis Spectrophotometer (METASH UV-5100). The reactions were stopped by adding an aqueous 2 mol/L of sodium carbonate (Na₂CO₃) solution (Baffi et al., 2011). An enzymatic activity unit was defined as the amount of enzyme required to release 1 μ mol of *p*NP from the substrate (PNPG) per min of reaction under the defined assay conditions. The β -xylosidase activity was determined by the same method, but with *p*-nitrophenyl- β -D-xylopyranoside (PNPX, Sigma) as substrate.

2.6 Exoglucanase, endoglucanase and xylanase

The activities of exoglucanase (avicelase), endoglucanase (carboxymethylcellulase - CMCase) and xylanase were investigated using 1.0% (w/v) of avicel, carboxymethylcellulose (CMC) and xylan as substrates, respectively, in sodium citrate buffer (0.1 mol/L, pH 4.8), for 10 min at 40 °C (Rodrigues et al., 2020). The reaction was stopped by the addition of 3,5-dinitrosalicylic acid (DNS) and boiled in boiling water bath for 10 min (Miller, 1959). Then, the samples were cooled in ice bath and 2.4 mL of distilled water was added. The amount of glucose/xylose released was measured at 540 nm. An enzymatic activity unit was defined as the amount of enzyme required to release 1 μ mol of β -D-glucose or β -D-xylose per min of reaction, determined from standard curves.

2.7 Filter paper cellulase

The FPase activity was determined using Whatman No. 1 filter paper (1.0 cm x 6.0 cm \cong 50 mg) in a reaction mixture containing 1 mL of 0.05 mol/L sodium citrate buffer (pH 4.8) and 0.5 mL of enzymatic extract at 50 °C for 1 h (Ghose, 1987). The reaction was interrupted by the addition of 3 mL of DNS and boiled for 5 min in boiling water bath. Then, the samples were cooled in an ice bath and homogenized with 20 mL of distilled water. The amount of glucose released was measured at 540 nm. The FPase activity unit (FPU) was defined as the amount of enzyme required to release 1.0 μ mol of β -D-glucose per min.

2.8 β -Glucosidase and β -Xylosidase Characterization

The optimum pH, optimum temperature, stability pH and thermostability of β -Glucosidase and β -xylosidase in the crude enzymatic extract from the consortium between *P. ostreatus* PLO6 and *A. niger* SCBM4 were determined in order to characterize this extract for future applications of biomass hydrolysis. The assays were performed in triplicate.

The optimum pH for each enzyme was assayed by incubating 50 μ L of crude enzymatic extract in 250 μ L of substrate (using PNPG and PNPX, respectively), varying the solution pH from 3.5 to 7.5 (with gradual increments of 0.5). The following buffer solutions were used: 0.1 mol/L sodium citrate buffer (pH 3.5 to 6.0) and 0.1 mol/L Tris-HCl buffer (6.5 and 7.5). The assays were performed at 40 °C for 10 min. The optimum temperatures were determined by incubating the reaction mixtures at the optimum pHs, under the same experimental conditions, at temperatures from 30 to 70 °C, with gradual increments of 5 °C (Dos Santos et al., 2015).

The stability pH was analyzed by incubating the crude enzymatic extracts for 24 h at room temperature diluted in the reported buffer solutions (pH from 3.5 to 7.5), without substrates. Thermostability was evaluated by incubating the enzyme extracts in the buffer solution at the optimum pH for each enzyme, for 1 h at temperatures from 30 to 70 °C. The residual activities (%) were determined at the respective optimum pHs and temperatures, under the same experimental conditions (Dos Santos et al., 2015).

3. Results and Discussion

3.1 Chemical Composition of the Substrates

Sugarcane bagasse was composed of 54.36% of cellulose, 13.52% of hemicelluloses and 26.14% of lignin (Table 1), indicating that the evaluated sample presents a lignocellulosic structure similar to those reported in previous studies (Rodrigues et al., 2017; Lamounier et al., 2020). This result showed SB as a rich source of polysaccharides for potential use in the biosynthesis of cellulases and hemicellulases. Wheat bran was composed of 25.41% cellulose, 26.45% hemicellulose and 25.77% lignin, also demonstrating its usefulness as a good substrate for the growth of fungi producers of (hemi)-cellulolytic enzymes by ssf. The results are in accordance with the characteristics of the residues obtained in previous studies (Dias et al., 2017).

Table 1. Chemical composition of carbon sources used in SSF of this study (SB: sugarcane bagasse; WB: wheat bran).

Composition (wt.%)	SB	WB
Moisture	9.53	9.4
Ash	0.65	1.76
Klason Lignin	26.14	25.77
Cellulose	54.36	25.41
Hemicellulose	13.52	26.95

The results are expressed as average \pm standard deviation (n = 3). Source: Authors.

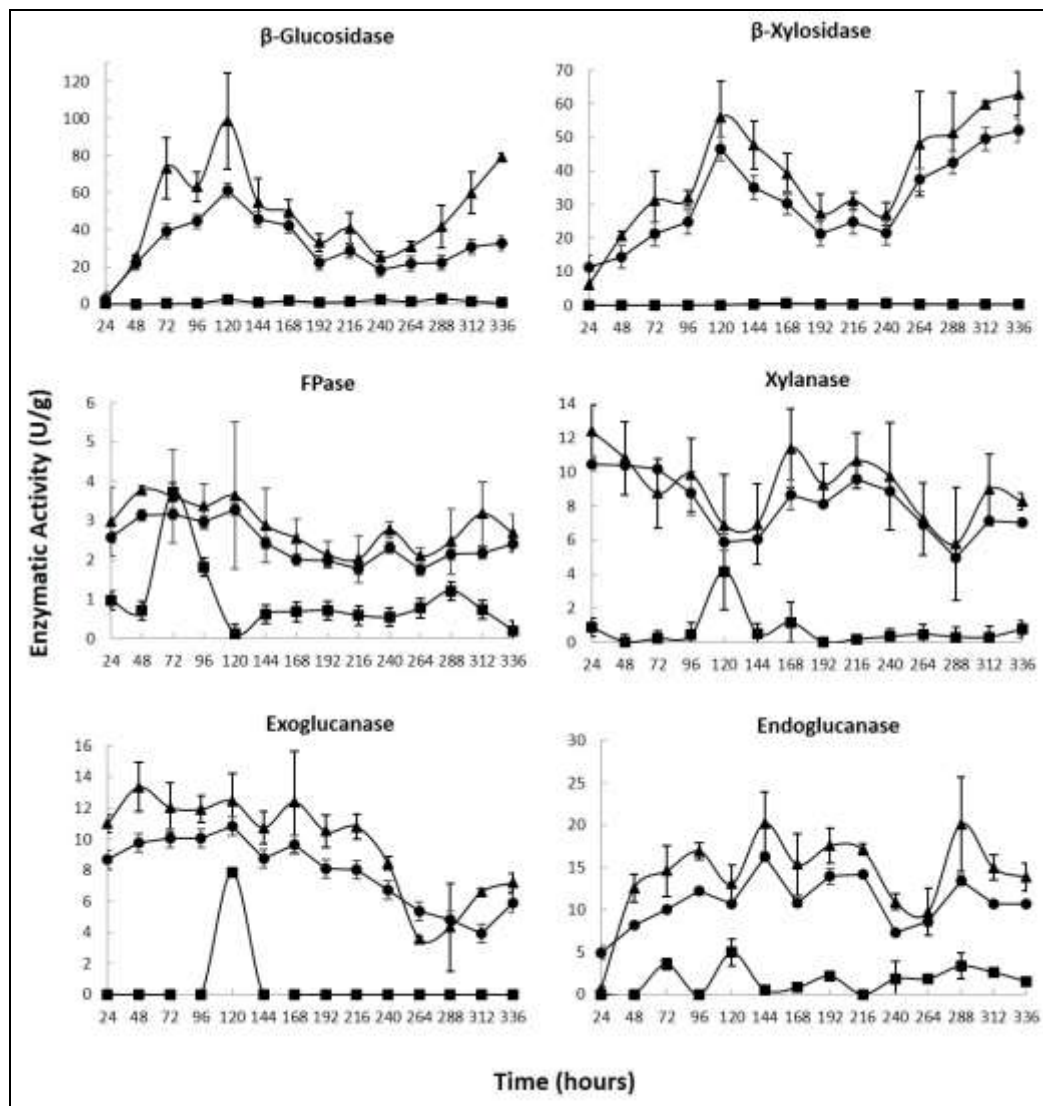
3.2 Enzymatic Production Profiles

After the SSF, the biosynthesis of cellulases and hemicellulases by the fungal strains cultured individually or in consortium was evaluated. Among all the evaluated conditions, the Fermentation 2, carried out with *P. ostreatus* PLO6 and *A. niger* SCBM4 consortium, exhibited the highest enzymatic productions with 98.51, 62.92, 3.77, 12.40, 13.37 and 20.19 U/g for β -glucosidase, β -xylosidase, FPase, xylanase, exoglucanase and endoglucanase, respectively (**Erro! Fonte de referência não encontrada.**). These results demonstrated that the fungal consortium (F2) stood out in the production of all enzymes, with increased activities in this crude extract in comparison to the enzymatic cocktails synthesized by *P. ostreatus* and *A. niger* monocultures. This result can be due to the tendency of microbial co-cultures in to overcome nutritional limitations, since they

can act synergistically on these complex substrates, degrading them more easily in monomers and leading to higher enzymatic yields than the isolated cultures (Lin et al., 2011; Sowmya et al., 2015). Previous studies have demonstrated that microbial consortia can be more efficient for the production of lignocellulose-degrading enzymes than fungal isolates (Singh et al., 2019; Rodrigues et al., 2020). Thus, the cultivation of the fungal strains in consortium in the present study can have contributed to the SSF process, becoming it more dynamic and filling some of the gaps that could limit the enzymatic biosynthesis by the monocultures.

Among the investigated enzymes, β -glucosidase was highlighted with maximum activity (98.51 U/g) after 120 h of fermentation (Figure 1). This result was superior than those obtained in some previous studies. For example, lower levels of β -glucosidase were observed by Dos Santos et al. (2015), who obtained 54 U/g of β -glucosidase by *A. niger* SCBM3 cultivated in monoculture and Dos Santos et al. (2019), who observed 80 U/g by the consortium between *A. niger* SCBM1 and *A. fumigatus* SCBM6, both with sugarcane bagasse and wheat bran as substrates. β -Xylosidase was the second enzyme with the highest production, presenting two peaks of activity, the first one also at 120 h of cultivation (56.03 U/g) and the second one (62.92 U/g) at 336 h. This result suggests the presence of two different kinds of β -xylosidases in this cocktail.

Figure 1. Time course of enzymatic production (U/g) by Solid State Fermentation (SSF) with *P. ostreatus* PLO6 and *A. niger* SCBM4 in monocultures or in consortium.



The results are expressed as average \pm standard deviation (n = 3). F1 (-■-: *P. ostreatus* PLO6 monoculture), F2 (-

▲-: *P. ostreatus* PLO6 and *A. niger* SCBM4 consortium) and F3 (-●-: *A. niger* SCBM4 monoculture). Source: Authors.

Regarding the production of cellulases and hemicellulases among strains of *P. ostreatus*, some studies observed that the biosynthesis of these enzymes by the same fungal species can vary depending on the substrate used in SSF (Shashirekha et al., 2005; Da Luz et al., 2012). Khalil et al. (2011) obtained nearby β -glucosidase and exoglucanase values (3.60 and 7.35 U/g, respectively) to the present study by SSF using *P. ostreatus* (Jacquin ex Fr.) and rice straw as carbon source for microbial growth. On the other hand, Kurt and Buyukalaca (2010) evaluated the production of CMCase using different agricultural residues by *P. ostreatus* HK 35 and obtained the highest activity of 2.20 U/g (an inferior value to the obtained in F1 from the present study) using wheat straw:bran (2:1) as substrates. These authors did not also obtain significant activities of hemicellulases at these conditions, suggesting low efficiency in the production of these enzymes by this species. However, Basidiomycetes from the genus *Pleurotus* are well known wood decomposers due to their ability to grow in lignocellulosic substrates (Mustafa et al., 2016; Arruda et al., 2021), being useful in processes of production of lignocellulose-degrading enzymes when cultured in association with Ascomycetes, such as *Aspergillus* species (Rodrigues et al., 2020; Zamora et al., 2021).

The present data are in agreement with prior studies that have verified remarkable production of enzymes from the (hemi)cellulolytic complex by species of the genus *Aspergillus*, chiefly *A. niger* (Khanahmadi et al., 2018; Rodrigues et al., 2020). For example, Rodrigues et al. (2017) obtained maximum productions of β -glucosidase (43.02 U/g) and β -xylosidase (78.00 U/g) by *A. niger* SCBM1 in monoculture, also using SB and WB as carbon sources. Recent studies have also demonstrated that the presence of the species *A. niger* among the inocula have increased the enzymatic production, reinforcing the importance of this species for the biosynthesis of cellulases and hemicellulases. Rodrigues et al. (2020) evaluated the enzymatic production by SSF, with raw sugarcane bagasse and raw wheat bran as substrates and fungal species cultured individually and in consortium (*Aspergillus fumigatus* SCBM6, *Aspergillus niger* SCBM1, *Ganoderma lucidum* 601, *Pleurotus ostreatus* PLO6 and *Trametes versicolor* 561). These authors observed that *Aspergillus* species, mainly *A. niger* were highlighted for cellulases and hemicellulases production, especially β -glucosidase and xylanases.

Dos Santos et al. (2019) also observed that the co-culture of *A. niger* SCBM1 and *A. fumigatus* SCBM6 was advantageous for the expression of β -xylosidase with maximum activity (180 U/g) after 48 h of SSF. At the same manner, in the present study, the co-cultivation of *A. niger* SCBM4 and *P. ostreatus* PLO6 demonstrated the enhancement of cellulases and hemicellulases biosynthesis, especially β -glucosidase and β -xylosidase, when compared to the fermentation carried out by these species in monocultures. These data indicated the great potential of this fungal consortium for the production of such enzymes, with fundamental role in the synergistic biomass degradation, through the hydrolysis of cellobiose and xylobiose, previously produced from the action of other cellulases and hemicellulases (Rodrigues et al. 2020). From the exposed, due to its greater effectiveness in the biosynthesis of enzymes, especially β -glucosidase and β -xylosidase (Table 2), the F2 cocktail with 120 h of microbial growth was selected to continue the study. Since β -glucosidase and β -xylosidase are key enzymes in the release of glucose and xylose, the effects of pH and temperature on their activities and stabilities in this extract were determined, aiming further applications.

Table 2. Comparison of enzymatic production (U/g) between F1 (*P. ostreatus* monoculture), F2 (*P. ostreatus* PLO6 and *A. niger* SCBM4) and F3 (*A. niger* SCBM4 monoculture). Enzymatic extracts of 120 h.

Enzyme	F1	F2	F3
β -glucosidase	2.95	98.51	61.14
β -xylosidase	0.54	62.92	46.54
FPase	3.72	3.77	3.28
Xylanase	4.15	12.40	5.85
Exoglucanase	7.89	13.37	10.84
Endoglucanase	4.95	20.19	10.69

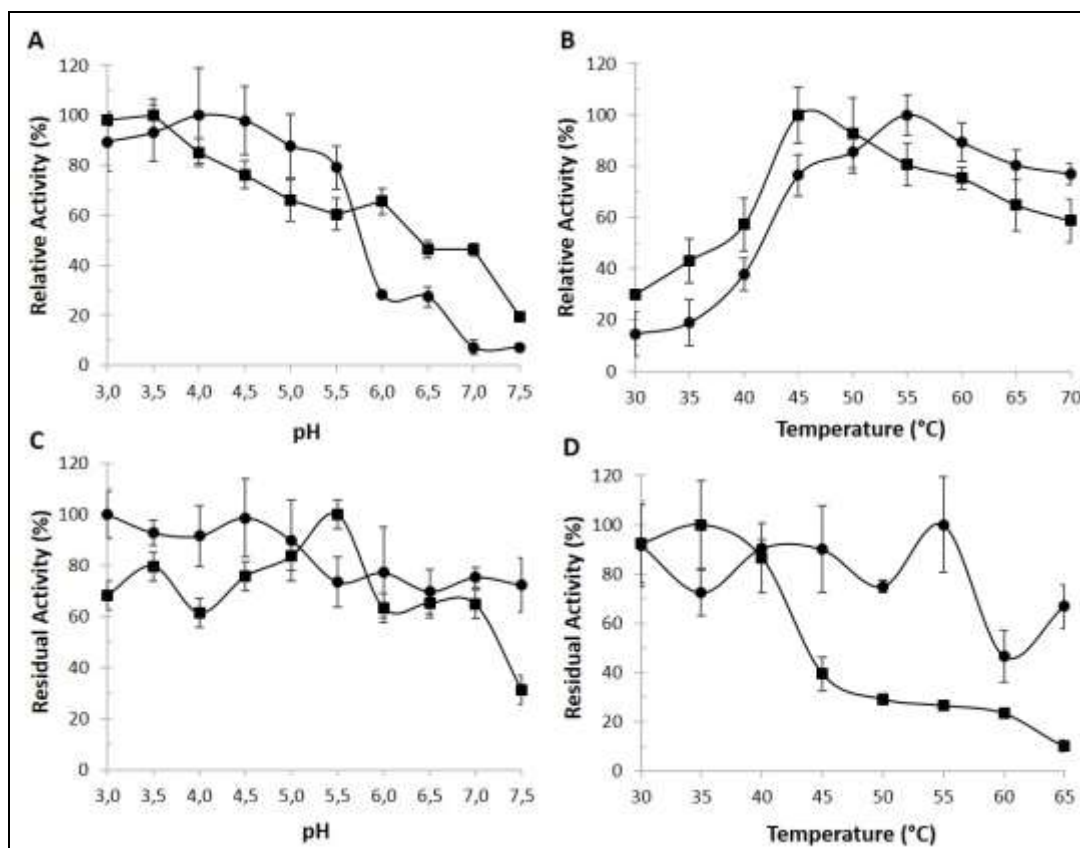
The values of activities were obtained from averages and standard deviations ($n = 3$) for each enzyme in each extract.
 Source: Authors.

3.3 Effects of pH and temperature on β -Glucosidase and β -xylosidase in F2 extract

The pH and the temperature are determining factors for the effectiveness of enzymatic saccharifications of lignocellulosic biomass since they can influence the enzymes activity due to the conformation changes and thermal denaturation (Nelson and Cox 2017). In this study, the β -glucosidase from F2 extract presented optimal activity at pH 4.0 and pH stability (above 60%) among 3.5 to 5.5, suggesting an acidophilic nature for this enzyme (**Erro! Fonte de referência não encontrada.**). Previous studies of β -glucosidase characterization produced by *A. niger* monocultures have reported maximum activities at pH ranges from 3.5 to 5.5, corroborating the present results (Rodrigues et al., 2017; Infanzón-Rodríguez et al., 2021). For example, Dos Santos et al. (2015) observed optimal activity at pH 3.5 for β -glucosidase from *A. niger* SCBM3, while Rodrigues et al. (2017) obtained optimum pH of 4.5 for β -glucosidase from *A. niger* SCBM1. In addition, according to earlier works, the majority of fungal β -glucosidases present optimal pH ranges from 4.0 to 6.0 and stability among 3.5-7.0 (Baffi et al, 2011; Almeida et al., 2021).

Regarding the temperature, the highest β -glucosidase activity was detected at 45 °C, achieving 99.74 U/g (**Erro! Fonte de referência não encontrada.**). Dos Santos et al. (2015) observed optimal activities at nearby temperature (50 °C) for a β -glucosidase produced by *A. niger* SCBM3 monoculture. For thermostability, the residual activity remained above 80% after incubation between 30 and 40 °C (**Erro! Fonte de referência não encontrada.**). Above 45 °C, the stability began to decrease, indicating thermal denaturation of the enzyme. Dos Santos et al. (2015) also observed thermostability up to 40 °C for β -glucosidase produced by *A. niger* SCBM3, corroborating the present data. Thus, the present results indicate that the β -glucosidase from the *P. ostreatus* PLO6 and *A. niger* SCBM4 crude extract comprised suitable properties for application in biomass hydrolysis, since this process is usually performed in acid pHs around 4.5 and temperatures of around 50 °C (Zain et al., 2018; Raj and Krishnan, 2019). For β -xylosidase, the optimum activity was verified at pH 3.5 and 55 °C, reaching 172.48 U/g (**Erro! Fonte de referência não encontrada.**A and 2B). In addition, it was observed that the enzymatic activity remained stable from pH 3.0 to 7.5 and among 30 to 55 °C. These results are in accordance with those reported by Dos Santos et al. (2015), who observed optimal activities at pH 3.0 and 55 °C and stability at pHs 4.0-7.0 and temperatures of 30-60 °C for β -xylosidase produced by *A. niger* SCBM3 monoculture.

Figure 2. Physicochemical characterization of β -glucosidase (\blacksquare -) and β -xylosidase (\bullet -) in F2 crude enzymatic extract (*P. ostreatus* PLO6 and *A. niger* SCBM4 consortium). A: Optimum pH, B: Optimum Temperature, C: pH Stability, D: Thermostability.



The results are expressed as average \pm standart deviation (n = 3). Source: Authors.

Fungal cellulases and hemicellulases from thermophilic strains are mostly tolerant to temperatures of up to 70 °C [7]. However, few enzymes can resist to elevated temperatures during long periods (Baffi et al. 2011; Rodrigues et al. 2017). This way, studies such as the present work are important to provide information of novel homemade enzymatic extracts which contain appropriate characteristics for use in hydrolytic processes wherein temperature changes may occur (Infanzón-Rodríguez et al. 2020). In this study, both β -glucosidase and β -xylosidase from the *P. ostreatus* PLO6 and *A. niger* SCBM4 extract exhibited stability at temperatures of up to 50 °C, indicating that this on-site cocktail presented promising characteristics for application in lignocelluloses hydrolysis which generally occur at temperatures around 45-50 °C (Raj and Krishnan 2019; Rodruigues et al., 2021).

4. Conclusion

This study showed the potential of the association between *P. ostreatus* PLO6 and *A. niger* SCBM4 for the production of important lignocellulose-degrading enzymes, with sugarcane bagasse and wheat bran as carbon sources. The enzymatic yields were higher in the cocktail produced by fungal consortium than by monocultures with significant increases, mainly β -glucosidase and β -xylosidase. β -Glucosidase was the enzyme that exhibited the highest production (99.74 U/g), with optimum activities at pH and temperature suitable for saccharifications (4.0 and 45 °C). Likewise, β -xylosidase presented maximum activities (172.48 U/g) in nearby pH and temperature values (3.5 and 55 °C) and both maintained stability in acid pHs and up to 50 °C. The findings stimulate the continuity of studies about the use of this enzymatic extract in processes of biomass

bioconversion, such as ethanol production. This work reinforces the importance of bioprospecting household enzymes, opening new perspectives for waste reuse and improvement of the viability of biorefineries. The effectiveness of the selected enzymatic extract in the hydrolysis of lignocellulosic biomass and sugar release will be evaluated in further studies.

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References

- Almeida, L. E. S., Ribeiro, G. C. A., & de Assis, S. A. (2021). β -Glucosidase produced by *Moniliophthora perniciosa*: Characterization and application in the hydrolysis of sugarcane bagasse. *Biotechnology and Applied Biochemistry*, 1–11.
- Araújo, N. L., Avelino, K. V., Halabura, M. I. W., Marim, R. A., Kassem, A. S. S., Santana, T. T., Colauto, G. A. L., Colauto, N. B., & Valle, J. S. do. (2021). Production of mycelial biomass and lignocellulolytic enzymes of *Pleurotus* spp. in liquid culture medium. *Research, Society and Development*, 10(1), e6810111406.
- Arruda, A. G., Evangelista, I. V., Menezes, L. S. De, Fischer, J., Cardoso, V. L., Santos, L. D., & Guidini, C. Z. (2021). Production of enzymatic complex from agro-industrial biomass and its application in combustible ethanol. *Research, Society and Development*, 10(6), e40410613705.
- Baffi, M. A., Tobal, T., Lago, J. H. G., Leite, R. S., Boscolo, M., Gomes, E., & Da Silva, R. (2011). A Novel β -Glucosidase from *Sporidiobolus pararoseus*: Characterization and Application in Winemaking. *Journal of Food Science*, 76(7), C997-C1002.
- Canilha, L., Chandel, A. K., Milessi, T. S. S., Antunes, F. A. F., Freitas, W. L. C., Felipe, M. G. A., & da Silva, S. S. (2012). Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. *Journal of Biomedicine and Biotechnology*, 7, 989572.
- Da Luz, J. M. R., Nunes, M. D., Paes, S. A., Torres, D. P., Silva, M. D. C. S. D., & Kasuya, M. C. M. (2012). Lignocellulolytic enzyme production of *Pleurotus ostreatus* growth in agro-industrial wastes. *Brazilian Journal of Microbiology*, 43(4), 1508-1515.
- Dias, L. M., dos Santos, B. V., Albuquerque, C. J. B., Baeta, B. E. L., Pasquini, D., Baffi, M. A. (2017). Biomass sorghum as a novel substrate in solid-state fermentation for the production of hemicellulases and cellulases by *Aspergillus niger* and *A. fumigatus*. *Journal of Applied Microbiology*, 124(3), 708-718.
- Dos Santos, B. V., Rodrigues, P. D. O., Albuquerque, C. J. B., Pasquini, D., & Baffi, M. A. (2019). Use of an (hemi) cellulolytic enzymatic extract produced by *Aspergilli* species consortium in the saccharification of biomass sorghum. *Applied Biochemistry and Biotechnology*, 189(1), 37-48.
- Dos Santos, B. S. L., Gomes, A. F. S., Franciscon, E. G., Oliveira, J. M. D., & Baffi, M. A. (2015). Thermotolerant and mesophilic fungi from sugarcane bagasse and their prospection for biomass-degrading enzyme production. *Brazilian Journal of Microbiology*, 46(3), 903-910.
- Ghose, T. K. (1987). Measurement of cellulase activities. *Pure Applied Chemistry*, 59, 695–702.
- Gomes, A. F. S., dos Santos, B. S. L., Franciscon, E. G., & Baffi, M. A. (2016). Substrate and temperature effect on xylanase production by *Aspergillus fumigatus* using low cost agricultural wastes. *Bioscience Journal*, 32, 915-921.
- Infanzón-Rodríguez, M. I., Ragazzo-Sánchez, J. A., del Moral, S. et al. (2020). Production and characterization of an enzyme extract with cellulase activity produced by an indigenous strain of *Fusarium verticillioides* ITV03 using sweet sorghum bagasse. *Biotechnology Letters*, 42, 2271–2283.
- Infanzón-Rodríguez, M. I., Ragazzo-Sánchez, J. A., Del Moral, S., Calderón-Santoyo, M., & Aguilar-Uscanga, M. G. (2021). Enzymatic hydrolysis of lignocellulosic biomass using native cellulase produced by *aspergillus niger* ITV02 under liquid state fermentation. *Biotechnology and Applied Biochemistry*, <https://doi.org/10.1002/bab.2097>.
- Khalil, M. I., Hoque, M. M., Basunia, M. A., Alam, N., & Khan M. A. (2011). Production of cellulase by *Pleurotus ostreatus* and *Pleurotus sajor-caju* in solid state fermentation of lignocellulosic biomass. *Turkish Journal of Agriculture and Forestry*, 35(4), 333-341.
- Khanahmadi, M., Arezi, I., Amiri, M., & Miranzadeh, M. (2018). Bioprocessing of agro-industrial residues for optimization of xylanase production by solid state fermentation in flask and tray bioreactor. *Biocatalysis Agricultural Biotechnology*, 13, 272-282.
- Kurt, S. & Buyukalaca, S. (2010). Yield performances and changes in enzyme activities of *Pleurotus* spp. (*P. ostreatus* and *P. sajor-caju*) cultivated on different agricultural wastes. *Bioresource Technology*, 101(9), 3164-3169.
- Lamounier, K. F. R., Rodrigues, P. O., Pasquini, D., & Baffi, M. A. (2020). Ethanol production and other bioproducts by *Galactomyces geotrichum* from sugarcane bagasse hydrolysate. *Current Microbiology*, 77,738–745.
- Lin, H., Wang, B., Zhuang, R., Zhou, Q., & Zhao, Y. (2011). Artificial construction and characterization of a fungal consortium that produces cellulolytic enzyme system with strong wheat straw saccharification. *Bioresource Technology*, 102(22), 10569-10576.

- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3), 426-428.
- & Mustafa, A. M., Poulsen, T. G., & Sheng, K. (2016). Fungal pretreatment of rice straw with *Pleurotus ostreatus* and *Trichoderma reesei* to enhance methane production under solid-state anaerobic digestion. *Applied Energy*, 180, 661-671.
- Nelson, D. L., & Cox, M. M. (2012). *Lehninger Principles of Biochemistry*, (6th ed.), W.H. Freeman.
- Raj, K. and Krishnan, C. (2019). Improved high solid loading enzymatic hydrolysis of low-temperature aqueous ammonia soaked sugarcane bagasse using laccase-mediator system and high concentration ethanol production, *Industrial Crops and Products*, 131, 32-40.
- Rani, V., Mohanram, S., Tiwari, R., Nain, L., & Arora, A. (2014). Beta-glucosidase: key enzyme in determining efficiency of cellulase and biomass hydrolysis. *Journal of Bioprocessing Biotechniques*, 5(1), 197-205.
- Rodrigues, P. D. O., Pereira, J. D. C., Santos, D. Q., Gurgel, L. V. A., Pasquini, D., & Baffi, M. A. (2017). Synergistic action of an *Aspergillus* (hemi-)cellulolytic consortium on sugarcane bagasse saccharification. *Industrial Crops and Products*, 109, 173-181.
- Rodrigues, P. D. O., Gurgel, L. V. A., Pasquini, D., Badotti, F., Goês-Neto, A., & Baffi, M. A. (2020). Lignocellulose-degrading enzymes production by solid-state fermentation through fungal consortium among Ascomycetes and Basidiomycetes. *Renewable Energy*, 145, 2683-2693.
- Rodrigues, P. D. O., Barreto, E. da S., Brandão, R. L., Gurgel, L. V. A., Pasquini, D., & Baffi, M. A. (2021). On-site produced enzyme cocktails for saccharification and ethanol production from sugarcane bagasse fractionated by hydrothermal and alkaline pretreatments. *Waste and Biomass Valorization*, <https://doi.org/10.1007/s12649-021-01499-7>.
- Shashirekha, M. N., Rajarathnam, S., & Bano, Z. (2005). Effects of supplementing rice straw growth substrate with cotton seeds on the analytical characteristics of the mushroom, *Pleurotus florida* (Block and Tsao). *Food Chemistry*, 92(2), 255-259.
- Singh, A., Jasso, R. M. R., Gonzalez-Gloria, K. D., Rosales, M., Cerda, R. B., Aguilar, C. N., Singhanian, R. R., & Ruiz, H. A. (2019). The enzyme biorefinery platform for advanced biofuels production. *Bioresource Technology Reports*, 7, 100257.
- Sowmya, H. V., Ramalingappa, B., Nayanashree, G., Thippeswamy, B., & Krishnappa, M. (2015). Polyethylene degradation by fungal consortium. *International Journal of Environmental Research*, 9(3), 823-830.
- Toquero, C., & Bolado, S. (2014). Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. *Bioresource Technology*, 157, 68-76.
- Verma, N., Kumar, V., & Bansal, M. C. (2020). Valorization of waste biomass in fermentative production of cellulases: a review. *Waste and Biomass Valorization*, 12(2):613-640.
- Zain, M. M., Mohammad, A. W., Harun, S., Fauzi, N. A., & Hairom, N. H. H. (2018). Synergistic effects on process parameters to enhance enzymatic hydrolysis of alkaline oil palm fronds, *Industrial Crops and Products*, 122, 617-626.
- Zamora, H. D. Z., Silva, T. A. L., Varão, L. H. R. Baffi, M. A., & Pasquini, D. (2021). Simultaneous production of cellulases, hemicellulases, and reducing sugars by *Pleurotus ostreatus* growth in one-pot solid state fermentation using *Alstroemeria* sp. waste. *Biomass Conversion and Biorefinery*, <https://doi.org/10.1007/s13399-021-01723-3>.