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Soybean Hulls as a Dual Substrate for in Situ Enzyme Production and Enzymatic Hydrolysis

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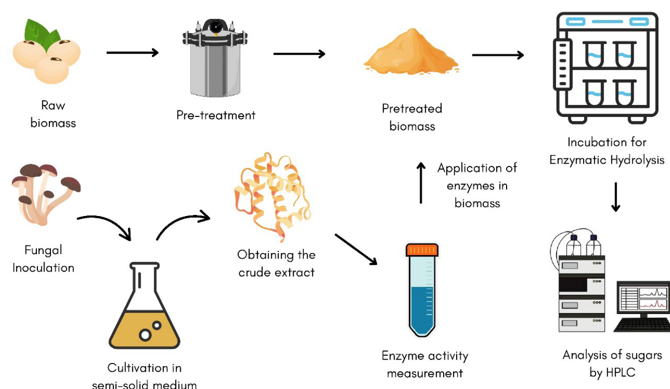
Keywords: Soybean hulls; Lignocellulolytic enzymes; Enzymatic hydrolysis; Phytopathogenic fungi.

Graphical Abstract



Abstract

The soybean hulls constitute an abundant and low-cost agricultural byproduct rich in polysaccharides. These compounds, in turn, are a promising substrate to produce sweeteners and biofuels via enzymatic hydrolysis. This work aimed to produce lignocellulolytic enzymes from three phytopathogenic fungi: *Nectriaceae* sp., *Cladosporium cladosporioides*, and *Phaeoacremonium parasiticum*, using the soybean hulls both as an inducer for cultivation and as a substrate for enzymatic hydrolysis. The enzymatic extracts were produced by semi-solid cultivation, using raw soybean hulls, for 8, 10, and 12 days. The biomass was subjected to two distinct pretreatments (hydrothermal with only distilled water, and acid with 0.5% H_2SO_4). Hydrolysis was conducted with 2.5 Filter Paper Units per gram (FPU/g) of pretreated hulls (10% solids) for 120 h at 50 C, using the commercial cocktail Multifect® CL Cocktail as a positive control. The enzymatic profile showed that *Nectriaceae sp.* was promptly discarded due to lack of activity, while *C. cladosporioides* stood out, achieving high specific activities for enzymes such as mannanase (80 U/mg), xylanase (43 U/mg), and α-galactosidase (27 U/mg). The hydrolysis results showed that the commercial cocktail released the highest concentration of glucose (2.222 g/L) in the acid-pretreated hulls, but only 0.452 g/L of xylose. In contrast, the extract produced by *C. cladosporioides*, when applied to hydrothermal-pretreated hulls, generated 1.007 g/L of glucose and 1.096 g/L of xylose, demonstrating an efficient and more balanced release of these sugars.

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Introduction

Soybeans are one of the world's most relevant agricultural commodities, playing a central role in global food and energy security. Soybean grain is used in the production of vegetable oil, protein for animal feed, and various industrial derivatives, making it essential for agribusiness [1]. In this context, Brazil stands out as the world's largest producer and exporter of soybeans, responsible for approximately 40% of global production. For the 2024/25 period, the Brazilian soybean harvest was estimated at 169.7 million tons, generating revenue of R$ 334.1 billion [2,3].

During the processing of the grain for oil extraction and meal production, large volumes of residues are generated, including the soybean hulls. Although often underutilized, this lignocellulosic biomass is rich in cellulose and hemicellulose, showing potential as a renewable raw material [4]. It is estimated that for every ton of processed soybean, 40 to 50 kg of hulls are obtained, representing an abundant and low-cost source for biotechnological applications [5]. This approach of valorizing residual biomass, such as the soybean hulls, is essential to produce second-generation biofuels through the fermentation of biomass sugars [6].

The utilization of soybean hulls depends on the deconstruction of their lignocellulosic matrix, a process that can benefit from the high catalytic specificity of lignocellulolytic enzymes, which function under mild conditions and minimize the formation of toxic byproducts that hinder fermentative processes [7]. However, due to the rigid and recalcitrant structure of the biomass, chemical pretreatment is necessary so that the enzymes can access the polysaccharides. Successful pretreatment strategies, which employ temperatures above 50 °C, are effective in disorganizing the biomass and facilitating enzymatic penetration [8].

Among the main enzymatic groups involved, the combination of cellulases and xylanases is considered the most effective in degrading lignocellulose [9]. Cellulases catalyze the depolymerization of cellulose and are composed of three enzymes: endoglucanases (EC 3.2.1.4), beta-glucosidases (EC 3.2.1.21), and exoglucanases (EC 3.2.1.91), which act in synergy to completely hydrolyze cellulose into glucose monomers [10]. Similarly, xylanases are essential for the hydrolysis of hemicellulose and comprise a set of enzymes: endo-1,4-beta-D-xylanases (EC 3.2.1.8), beta-D-xylosidases (EC 3.2.1.37), feruloyl-esterases (EC 3.1.1.73), acetylxylan-esterases (EC 3.1.1.72), alpha-glucuronidases (EC 3.2.1.139), alpha-L-arabinofuranosidases (EC 3.2.1.55), and p-coumaric esterases (EC 3.1.1.B10). These enzymes work together to degrade xylan from hemicellulose into monosaccharides and xylo-oligosaccharides [11].

Fungi, due to their natural capacity to secrete extracellular enzymes, have been widely explored as producers of these lignocellulolytic enzymes [12]. Among them, phytopathogenic fungi stand out for their ability to degrade the plant cell wall during infection, efficiently producing enzymes such as cellulases, xylanases, and laccases [13]. Despite this potential, their use for lignocellulases production is still limited and little studied compared to non-pathogenic fungi, such as those of the genera *Trichoderma* and *Aspergillus* [14].

In this scenario, the soybean hulls assume a dual and strategic role in biotechnological processes. It not only serves as a raw material for enzymatic hydrolysis, generating fermentable sugars, but it can also function as an inducing substrate to produce lignocellulolytic enzymes by microorganisms. This capacity to act on both fronts, as a carbon source for enzyme production and as an input for saccharification, makes it a biomass of great interest in the production of biofuels [15].

Thus, the use of soybean hulls as a substrate to produce fermentable sugars through enzymatic hydrolysis emerges as a sustainable and economically attractive strategy. In addition to adding value to a low-cost agro-industrial residue, this process fits within the context of the circular economy, contributing to waste reduction, mitigation of environmental impacts, and the generation of essential inputs for strategic sectors such as energy and food [16].

Methodology

Pretreatment of soybean hulls

The soybean hulls samples, supplied by the company CJ Selecta, were dried in an oven at 70 °C until constant weight was achieved, and subsequently ground in a knife mill to particles smaller than 1 mm. The hulls were subjected to acid and hydrothermal pretreatments, using a 0.5% (w/v) H_2SO_4 solution for acid pretreatment and only water for hydrothermal treatment. The pretreatments were carried out in an autoclave at 120 °C for 30 min, with a solid concentration of 10% (w/v). Subsequently, the obtained solution was filtered by vacuum filtration and the retained solid was dried in an oven at 50 °C.

Determination of inhibitor compounds after pretreatment

The determination of acetic acid, formic acid, furfural, and 5-Hydroxymethyl-2-Furaldehyde (HMF) contents were performed by HPLC with an HPX-87H column (300×7.8 mm), maintained at 60 °C and 5 mM sulfuric acid eluent with a flow rate of 0.6 mL/min. The concentration of total phenolic compounds was determined by the Prussian blue method [17].

Cultivation of phytopathogenic fungi

The phytopathogenic fungi *Nectriaceae sp.*, *Cladosporium cladosporioides*, and *Phaeoacremonium parasiticum* were obtained from the mycological collection of the Forest Pathology Laboratory at UFV and were maintained at 28 °C on PDA (Potato Dextrose Agar) plates.

These fungi were cultivated in a semi-solid medium containing soybean hulls as a carbon source: 5 g in 12 mL of cultivation medium with the following composition, in g/L: NH_4NO_3 1.0; KH2PO4 1.5; $MgSO_4$; $CuSO_4$ 0.25; yeast extract 2.0 and trace elements, in mg/L: $MnCl_2$ 0.1; H_3BO_3 0.075; Na_2MoO_4 0.02; $FeCl_3$ 1.0; $ZnSO_4$ 3.5. The medium was inoculated with 10 discs of approximately 1.5 cm, obtained from the plates containing the fungi, and incubated for 8, 10, and 12 days at 150 rpm and 28 °C.

Extraction of cellulases and hemicellulases

The enzymes secreted by the phytopathogenic fungi during cultivation in semi-solid medium were extracted with 50 mM, pH 5 sodium acetate buffer, at a ratio of 10:1 (buffer: substrate mass), under stirring at 150 rpm for 60 minutes at room temperature. The crude extracts were obtained by vacuum filtration.

Determination of enzymatic activities

The crude extracts obtained after 8, 10, and 12 days were subjected to different assays for the determination of enzymatic activities. The assays were performed using 100 mM, pH 5.0 sodium acetate buffer and a temperature of 50 °C, for different times.

For assays based on the quantification of total reducing sugars, the 3,5-Dinitrosalicylic acid (DNS) colorimetric method was used [18]. A standard curve for reducing sugars was prepared using a glucose solution of 2 g/L. Serial dilutions were made, and the standards were subjected to the same DNS reaction procedure as the enzymatic assays, with absorbance measured at 540 nm to quantify the released product in µmol of glucose.

FPase (total cellulase) activity was determined using Whatman No. 1 filter paper (1×6 cm) as the substrate for 60 minutes. For endoglucanase, mannanase, and pectinase assays, the substrates were 1.25% (w/v) carboxymethylcellulose (CMC), 0.4% (w/v) locust bean gum, and 0.25% (w/v) polygalacturonic acid, respectively, for 30 minutes. Xylanase activity was determined using 1.0% (w/v) beechwood xylan for 15 minutes.

The colorimetric assays using synthetic substrates containing para-nitrophenol (pNP) were employed for the determination of α-glucosidase, β-glucosidase, β-xylosidase, α-galactosidase, β-galactosidase, α-arabinofuranosidase, and β-cellobiohydrolase activities, respectively, for 15 minutes in a water bath. A standard curve for pNP was constructed using a solution of pNP 2 µmol/mL. Aliquots of this standard were diluted in 100 mM, pH 5.0 sodium acetate buffer, and the reaction was stopped by adding an alkaline solution (e.g., Na_2CO_3) to develop the color, with absorbance measured at 405 nm for the quantification of released pNP in µmol.

For the determination of laccase activity, the substrate ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)) was used, incubating in a water bath for 15 minutes. The activity was quantified by spectrophotometry at 420 nm, and the conversion of absorbance to product concentration was performed using the Lambert-Beer Law.

The assays were performed in triplicate for the calculation of means and standard deviation. The unit of enzymatic activity was defined as U/mL, where U corresponds to the amount of enzyme that releases 1 µmol of product per minute.

Determination of protein content

The determination of the protein concentration of the different crude enzymatic extracts was performed by the Bradford method [19].

Enzymatic hydrolysis of pretreated soybean hulls

The enzymatic extracts from the phytopathogenic fungi *Cladosporium cladosporioides* and *Phaeoacremonium parasiticum* were selected for saccharification experiments, and the choice was based on the enzymatic profile of these extracts. Furthermore, a 1:1 mixture of the two enzymatic extracts was tested.

The enzymatic extracts were applied to the saccharification of pretreated soybean hulls, at 50°C, 250 rpm, for a period of 120 hours. The reaction volume consisted of 5% pretreated soybean hulls and fungal extract containing 2.5 FPase units Per gram of biomass (FPU/g), plus 10 mM sodium azide and 40 µg/mL tetracycline to prevent microbial contamination. For the positive control of the reaction, the commercial cocktail Multifect® CL Cocktail (Genencor Intl., USA) was used, and for the negative control, 50 mM, pH 5.0 sodium acetate buffer was used in the same proportions as the fungal extract. Each assay was performed in reaction triplicates. Aliquots were taken at different incubation times: 0, 24, 48, 72, 96, and 120 hours.

Quantification of glucose and xylose release after enzymatic hydrolysis

The aliquots derived from the saccharification experiment were analyzed by HPLC for the quantification of the released glucose and xylose sugars. A Shimadzu 10A series instrument with a refractive index detector was used, as described by Falkoski et al. (2013). The standard curve for quantification was prepared using solutions of glucose and xylose, both at 5 g/L. These solutions were serially diluted to cover the expected concentration range of the enzymatic saccharification samples. The column used in the HPLC was the Aminex HPX-87P (300×7.8 mm). Elution was performed with water, at a flow rate of 0.6 mL/min, at 80°C [20].

Statistical analysis

All data were statistically analyzed using one-way Analysis of Variance (ANOVA). Where significant differences were identified ($p<0.05$), Tukey's HSD test was subsequently applied for mean separation. All statistical analyses were performed using the R software [21].

Results and discussion

Enzymatic profile of fungal extracts

After cultivation in semi-solid medium, the profile of lignocellulolytic enzymes secreted by each fungus, when induced on soybean hulls, was determined. Table 1 shows the protein contents of the crude enzymatic extracts obtained after growth of the phytopathogenic fungi *Nectriaceae sp.*, *C. cladosporioides* and *P. parasiticum* on soybean hulls after 8, 10, and 12 days of cultivation. It is noticed that the protein content of the crude extracts obtained is quite similar, and the concentrations were close to 0.2 mg of protein per mL of enzymatic extract.

Table 2 details the specific enzyme activities for 11 different enzymes obtained from the crude extracts of the phytopathogenic *fungi Nectriaceae sp., C. cladosporioides, and P. parasiticum* following 8, 10, and 12 days of cultivation on soybean hulls. The overall fungal enzymatic profiles showed considerable variability. It is important to note that α-glucosidase and cellobiohydrolase activities were not detected across the samples. Based on the initial screening results, the extract from *Nectriaceae* sp. was immediately discarded from subsequent steps due to its near-total lack of activity across the tested enzymes.

In contrast, *C. cladosporioides* exhibited notably high activities across multiple enzymes, particularly emphasizing mannanase, β-glucosidase, and β-galactosidase. This fungus stood out as the top secretor for several key enzymes, achieving maximum activities after 12 days of cultivation. Specific peak values include mannanase (80 U/mg), xylanase (43 U/mg), α-galactosidase (27 U/mg), endoglucanase (19 U/mg), pectinase (16 U/mg), and β-glucosidase (6 U/mg).

Despite the 12 days of cultivation showing superior results compared to 8 and 10 days for *C. cladosporioides*, it was not possible to find a significant difference by ANOVA between the different times for most of the enzymes investigated. However, a statistically significant difference was found for a few specific enzymes in the 12-day extract. In this regard, for the hydrolysis steps, it was decided to produce and use the extract in the shortest possible time, i.e., 8 days of cultivation for both *C. cladosporioides* and *P. parasiticum*.

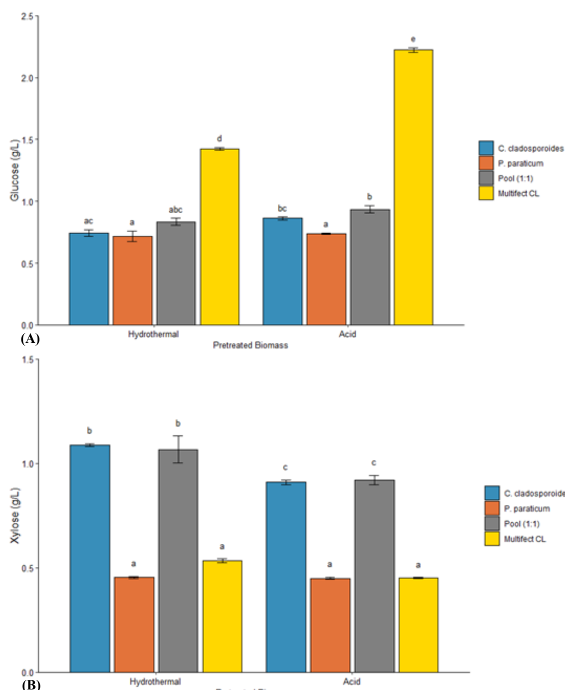

Figure 1: **(A)** Glucose and **(B)** xylose concentration (g/L) released after enzymatic hydrolysis of the pretreated biomass using enzymes from the fungi *C. cladosporioides* (blue), *P. parasiticum* (red), a 1:1 mixture of *C. cladosporioide* and *P. parasiticum* extracts (gray), and the commercial cocktail Multifect® CL Cocktail (yellow). Bars with different letters indicate statistically significant differences (Tukey's test, p<0.05).

Table 1: Protein content of crude extracts produced by the fungi after cultivation for different times on soybean husk. Data are presented as the mean ± standard deviation (n=6). The statistical comparison among all groups in each enzyme was performed using Analysis of Variance (ANOVA) followed by the Tukey's HSD test (p<0.05). Different uppercase letters (A, B, C, ...) indicate statistically significant differences between the tested groups.

Fungus	Cultivation Time (days)	Protein content (mg/mL)
C.cladosporioides	8	0.235±0.01 ABC
	10	0.217±0.02 ABC
	12	0.184±0.08 BC
P. parasiticum	8	0.205±0.07 ABC
	10	0.186±0.03 BC
	12	0.170±0.01 C
***Nectriaceae* sp.**	8	0.237±0.01 ABC
	10	0.262±0.01 A
	12	0.248±0.02 AB

Table 3: Inhibitors produced during the different pretreatments (mg/mL).

Inhibitors / Pretreatments	Formic Acid	Acetic Acid	Hydroxymethylfurfural	Furfural
Hydrothermal	0.085±0.010	0.122±0.012	ND	ND
Acid	0.283±0.011	ND	0.045±0,001	ND

Table 2: Specific enzyme activities (U/mg) of different enzymes produced by different fungi cultivated for 8, 10, and 12 days. Data are presented as the mean±standard deviation (n=6). Different letters indicate statistically significant differences (Tukey's test, p<0.05) between the groups of each enzyme. Activity results for α-glucosidase and β-cellobiohydrolase are not presented as activity was below the detection limit in all samples. ND, Not Detected; FPase, Filter Paper Activity.

Fungus	*C. cladosporoides*			*P. parasiticum*			*Nectriaceae sp.*		
Cultivation Time (days) / Enzymatic Activity	8	10	12	8	10	12	8	10	12
β-glucosidase	4.49±0.44 B	4.04±0.55 B	5.80±0.59 A	0.06±0.01 C	0.05±0.01 C	0.05±0.01 C	0.14±0.01 C	0.14±0.01 C	0.12±0.00 C
α-galactosidase	13.01±0.72 B	12.98±2.33 B	27.54±3.11 A	0.26±0.02 C	0.23±0.02 C	0.28±0.03 C	ND	ND	ND
β-galactosidase	0.12±0.00 A	0.12±0.03 A	0.08±0.01 A	ND	ND	ND	ND	ND	ND
β-xylosidase	0.10±0.00 A	0.12±0.02 A	0.05±0.00 B	ND	ND	ND	ND	ND	ND
α-arabinofuranosidase	0.38±0.02 BC	0.38±0.04 BC	0.27±0.02 C	0.47±0.08 AB	0.44±0.05 ABC	0.59±0.03 A	ND	ND	ND
FPase	2.30±0.11 A	2.48±0.29 A	1.87±0.08 AB	2.43±0.39 A	2.11±0.19 A	2.22±0.27 A	0.98±0.08 B	0.98±0.12 B	1.04±0.06 B
Mannanase	49.93±2.42 B	57.60±2.13 B	80.04±11.21 A	3.06±0.53 C	2.98±0.35 C	2.58±0.14 C	ND	ND	ND
Xylanase	23.48±1.37 B	23.06±2.67 B	43.43±4.37 A	29.10±4.77 B	19.13±1.46 B	24.80±2.05 B	ND	ND	ND
Endoglucanase	8.68±0.40 B	10.87±0.27 B	19.37±2.44 A	2.80±0.44 C	3.32±0.17 C	3.92±0.23 C	ND	ND	ND
Pectinase	13.19±0.37 AB	14.79±0.81 AB	16.13±1.59 A	11.40±2.36 AB	10.34±1.76 AB	7.05±2.09 B	ND	ND	ND
Laccase (10-4)	ND	ND	ND	ND	ND	ND	18.45±4.37 B	34.46±7.54 AB	47.05±7.93 A

Inhibitors produced in pretreatment

Table 3 shows the results of inhibitors quantification during the different pretreatments: hydrothermal and acid.

From these results, it can be observed that the hydrothermal and acid pretreatments produced formic acid and acetic acid in measurable concentrations, with the acid pretreatment yielding the highest concentration of formic acid (0.283±0.011 mg/mL). Hydroxymethylfurfural (HMF) was quantified exclusively after the acid pretreatment, in a small concentration (0.045±0.001 mg/mL). Phenolic compounds were not found as inhibitors produced by either pretreatment. This result is very interesting because phenolic compounds are described as the most inhibitory, potentially culminating in the precipitation and irreversible inhibition of enzymes [22].

Enzymatic hydrolysis

Figure 1 shows the concentration of glucose (g/L) (Figure 1A) and xylose (g/L) (Figure 1B), respectively, after 120 hours of enzymatic hydrolysis of the soybean hulls under different conditions.

From these results, it can be observed that the glucose release by the fungal enzymatic extracts was inferior to that performed by the commercial Multifect® CL Cocktail, which was expected as this cocktail is rich in cellulases. The mixture of enzymatic extracts did not result in a statistically significant increase in glucose release over the individual extracts of *C. cladosporioides* and *P. parasiticum.*

Both the hydrothermal and acid pretreatments yielded similar glucose release levels with the fungal enzymatic extracts, except for the commercial cocktail, which showed a greater release when applied to the acid-pretreated biomass.

Based on these results, both the extract from the fungus *C. cladosporioides* and the mixture of enzymatic extracts demonstrated superior xylose release compared to the Commercial Multifect® CL Cocktail, achieving up to a 2-fold increase.

However, the absence of a synergistic advantage in the mixture compared to the individual extracts, combined with the observation that the *C. cladosporioides* extract exhibited activity like the mixture, confirms its role as the predominant contributor to the mixture's overall enzyme activity.

Furthermore, xylose release was consistently higher when the enzymatic extracts were applied to the hydrothermally pretreated biomass as opposed to the acid-pretreated biomass.

Conclusion

Soybean hulls proved to be an efficient and low-cost inducer for the *in-situ* production of lignocellulolytic enzymes by the phytopathogenic fungi *C. cladosporioides* and *P. parasiticum*. The resulting fungal enzymatic extracts were shown to be promising by efficiently hydrolyzing the different pretreated soybean hulls samples, with a particular emphasis on xylose release. These results establish both soybean hulls (as a substrate) and the fungal extracts (as enzyme sources) as viable candidates for the low-cost production of specific enzymes and for the saccharification of biomass, aiming at the production of second-generation ethanol.

Author declarations

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