FUNGAL ENZYMES FOR SOY HUSK HYDROLYSIS AIMING ETHANOL PRODUCTION

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ABSTRACT

Second-generation ethanol can be produced from various agricultural wastes such as soy husk, which is widely available worldwide. To make its production feasible, low-costs pretreatments and enzyme cocktails must be applied. Fungal enzymes produced on-site, using lignocellulosic biomasses as inducers is a great alternative. This work aimed to use wheat bran, soy husk, and sugarcane bagasse to cultivate five different fungi and evaluate their enzymatic profile, focusing on pretreated soy husk enzymatic hydrolysis. Mild acid pretreatment had the biggest impact on sugars release by the commercial mixture Cellic® CTec3 HS, with 64.07 % of cellulose conversion, as well as a release of 7.10 g/L xylose, indicating hemicellulose depolymerization. Four fungal extracts were selected to be tested on acid-pretreated soy husk hydrolysis: *Aspergillus sydowii, Chrysoporthe cubensis* and *Talaromyces pinophilus*, cultivated in soy husk, and *C. cubensis*, cultivated in wheat bran. The extracts of *T. pinophilus* and *C. cubensis* cultivated in soy husk demonstrated the best rates of glucose release, 2.42 and 1.75 g/L, respectively. They were then combined, to evaluate synergistic effects. The blended extract was able to increase the hydrolysis rate, making glucose and xylose release up to 2.95 and 3.70 g/L, respectively.

Keywords: Fungi. Enzymes. Saccharification. Second-generation ethanol. Soy husk.

1 INTRODUCTION

Biorefineries, which convert biomass into energy, materials, and chemicals with higher economic value, are increasing globally, as an opposition to the previously dominant fossil fuel industry. They contribute to minimizing environmental impacts and creating more sustainable and economically reliable production chains [1]. Second-generation ethanol (2G ethanol), for instance, can be produced utilizing a series of agricultural wastes also called lignocellulosic biomasses as raw materials. Sugarcane and corn are widely used for ethanol production due to their elevated sugar content. However, with the rise of 2G ethanol, several other sources can be used, such as straws, bagasse, husks, cobs and stover [2]. Soybean is a highly cultivated crop, and Brazil, The United States, Argentina, and China leading its production ranks. These four countries alone, produce more than 300 million tons of soybean annually, which generates vast amounts of soy husk, which offers a great potential to be converted into ethanol [3]. Lignocellulosic biomasses are mainly composed of plant cell wall, a complex structure of the polysaccharides cellulose and hemicellulose intertwined with lignin [4]. Thus, 2G ethanol is not as straightforward as first-generation (1G) counterpart. Before fermentation, pretreatment and enzymatic hydrolysis must be performed, to deconstruct the recalcitrant structure, releasing fermentable sugars. Therefore, cost-effective enzyme production poses the main bottleneck for a feasible 2G ethanol [5]. For cellulose hydrolysis, which is formed by a series of glucose molecules, a small set of enzymes is necessary, mainly endoglucanase, celobiohydrolase, and β-glucosidase. On the other hand, hemicellulose is a complex polymer, with different backbones and substituents. Xylan, the most common hemicellulose, is constituted of a xylose backbone bonded with arabinose, galactose, acetyl groups, ferulic and galacturonic acids, among others. For this reason, xylanase, β-xylosidase, and a series of accessory enzymes act together in hemicelluloses breakdown [6]. There are several sources of enzymes, however, microorganisms are commonly used, for their rapid cultivation and simple extraction process. Fungi are especially targeted, for their ability to grow in a variety of conditions and they usually secrete the enzymes [7]. Semi-solid fungi cultivation shows many advantages when applied to biomass conversion: less water is necessary, which is highly important industrially, and agricultural wastes can be used as sources to induce the production of specific enzymes to depolymerize cellulose and hemicellulose [8]. Hence, soy husk presents a lot of potential to be used as raw material for ethanol production, as well as a carbon source to produce on-site enzymes, reducing the overall costs of the process [9]. This work aimed the production of enzymatic cocktails by different fungi, cultivated in semi-solid media, using wheat bran, sugarcane bagasse, and soy husk as carbon sources, to induce a diverse range of lignocellulolytic enzymes, focusing on pretreated soy husk hydrolysis, releasing sugars destinated to ethanol production.

2 MATERIAL & METHODS

2.1 Soy husk chemical composition and pretreatments

Cellulose, hemicellulose, lignin, proteins, extractives, and ash content of the soy husks, with and without pretreatments, were estimated as described by the National Renewable Energy Laboratory – NREL [10]. Mild hydrothermal, alkaline (NaOH 1.0 %) and acid (H_2SO_4 0.5 %) pretreatments were performed on soy husk, with 1:4 (m/v) solid ratio, at 120 °C, for 30 minutes. After, samples were extensively washed and filtered through a nylon cloth.

2.2 Fungi cultivation and enzymatic extract production

Ten mycelium disks of *Aspergillus sydowii, Chrysoporthe cubensis, Hypoxylon* sp., *Kretzschmaria zonata*, and *Talaromyces pinophilus* were cultivated for 8 days in 5 g of wheat bran, sugarcane bagasse or soy husk as carbon sources, at 28 °C, in 60 % humidity semi-solid media containing mineral media (NH_4NO_3 1.0 g/L; KH_2PO_4 1.5 g/L; $MgSO_4$ 0.5 g/L; $CuSO_4$ 0.25 g/L; yeast extract 2.0 g/L) and trace elements ($MnCl_2$ 0.1 mg/L; H_3BO_3 0.075 mg/L; Na_2MoO_4 0.02 mg/L; $FeCl_3$ 1.0 mg/L; $ZnSO_4$ 3.5 mg/L). Enzymes extraction was made under agitation at 150 rpm, with 50 mL of sodium citrate buffer 50 mM, pH 5.0 at room temperature for 1 h. Then, the extracts were filtered in nylon and centrifuged at 10000 g, 4 °C for 15 min.

2.3 Enzymatic profile and protein concentration

Different colorimetric methods were used to analyze the activity of 12 different enzymes. Assays based on reducing sugars release were performed with 3,5-dinitrosalicylic acid – DNS [11]. Synthetic *p*-nitrophenyl (*p*NP) based assays were performed using 50 µL of extract and substrate, and Na_2CO_3 0.5 M to stop the reaction. Laccase activity was analyzed by the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) - ABTS. All assays were performed at 50 °C, spectrophotometrically analyzed and compared to previously made analytical curves. Spectrometry analysis was performed at different wavelengths for different methods: 540 nm for DNS, 410 nm for pNP and 420 nm for the laccase assay. One activity unit (U) was established as the amount of enzyme capable of converting 1 µmol of product per minute. Enzymes, substrates, reaction times, and other parameters are shown in Table 1. Protein concentration followed with Bradford reagent [12].

Table 1 Enzymatic profile assays and procedure specifications .

Enzyme	Substrate	Concentration	Reaction time (min)	Method
endoglucanase	carboxymethyl cellulose	1.25 % (m/v)	30	DNS
mannanase	locust bean gum	1.25 % (m/v)	30	DNS
pectinase	polygalacturonic acid	0.25 % (m/v)	30	DNS
xylanase	xylan beechwood	1.25 % (m/v)	15	DNS
FPase	Whatmann no1 filter paper (1x6 cm)	-	60	DNS
α-glucosidase	pNP-α-D-glucopyranoside	2 mM	15	*p*NP
β-glucosidase	pNP-β-D-glucopyranoside	2 mM	15	*p*NP
β-xylosidase	pNP-β-D-xylopyranoside	2 mM	15	*p*NP
α-galactosidase	pNP-α-D-galactopyranoside	2 mM	15	*p*NP
β-glalactosidase	pNP-β-D-galactopyranoside	2 mM	15	*p*NP
α-arabinofuranosidase	pNP-α-D-arabinoopyranoside	2 mM	15	*p*NP
β-cellobiohydrolase	pNP-β-D-cellobiopyranoside	2 mM	15	*p*NP
Laccase	ABTS	10 mM	15	-

2.4 Enzymatic hydrolysis and sugars analyzes

Saccharification assays were carried out with the best potential extracts, using the commercial cocktail Cellic® CTec3 HS (Novozymes) as a positive control, following the proportions of 5 % soy husk (m/v), enzyme load of 2.5 FPU/g of biomass. Sodium azide 10 mM and tetracycline 40 µg/mL were added to avoid contamination. The final volume was completed with a citrate buffer 100 mM pH 5.0. The assay was carried for 120 h, at 50 °C, under agitation at 200 rpm, with aliquots taken each 24 h. Release of glucose and xylose was analyzed by High-Performance Liquid Chromatography – HPLC (CBM-20A/20Alite - Shimadzu). The sugars were separated by an Aminex HPX-87H column (BioRad) and detected by a refractive index detector (RID-20A – Shimadzu).

2.4 Statistical analyzes

The statistical analyzes were conducted to compare the data of enzymatic profiles and enzymatic hydrolysis of soy husk to verify if the means were statistically different. For this purpose, ANOVA and Tukey's Test (*p*-value < 0.05) were applied with the assistance of Minitab® 19.1 software.

3 RESULTS & DISCUSSION

The pretreatments were made under mild conditions, to avoid the formation of enzyme and fermentation inhibitors. Table 2 shows some changes in the composition of *in natura* and pretreated soy husk.

Table 2 Chemical composition of soy husk.

	Cellulose (%)	Hemicellulose (%)
In natura	36.26 ± 0.86	24.08 ± 0.50
Pretreated		
Hydrothermal	35.20 ± 0.53	24.05 ± 1.05
Alkaline NaOH 1.0 %	38.30 ± 0.79	21.14 ± 0.49
Acid H_2SO_4 0.5 %	38.13 ± 1.84	21.40 ± 1.11

Hydrothermal pretreatment had little impact on cellulose and hemicellulose content. When alkaline and acid pretreatments were compared, ANOVA analysis demonstrated no significant difference between their means. Thus, to better evaluate their impacts on the biomass, enzymatic hydrolysis with Cellic® CTec3 HS was performed. After the process, hydrothermal pretreatment was the least efficient with only 26.96 % cellulose conversion. Acid pretreatment was slightly better than the alkaline, comparing glucose generation, with 64.07 and 62.11 % of cellulose conversion, respectively. However, when xylose generation was evaluated, hydrolysis of the acid pretreated soy husk released 7.10 g/L and alkaline 5.33 g/L, suggesting higher hemicellulose conversion. For this reason, the 0.5 % H_2SO_4 mild pretreatment was selected for the next steps.

The enzymatic profile of the 5 fungi cultivated in wheat bran, sugarcane bagasse and soy husk highlighted the potential of soy husk as a good carbon source. All fungi grown in soy husk demonstrated higher activity levels in most of the tested enzymes, indicating its advantages as a cheap and widely available biomass. After performing ANOVA analysis and Tukey's test on the extracts, *A. sydowii, C. cubensis* and *T. pinophilus* cultivated in soy husk and *C. cubensis* cultivated in wheat bran were selected, for presenting the highest enzyme activity values in a diverse range of assays. These extracts were then utilized to hydrolyze acid pretreated soy husk to evaluate glucose and xylose release. *T. pinophilus* and *C. cubensis* in soy husk were the most efficient, with 2.42 and 1.75 g/L glucose, and 2.55 and 2.08 g/L xylose, respectively.

These two extracts were combined in a new hydrolysis assay to verify whether their combined enzyme arsenal would be more efficient or maintain the same hydrolysis yield. When combined, they were able to increase fermentable sugars release, suggesting a synergistic effect, making glucose and xylose release up to 2.95 and 3.70 g/L, respectively. Considering that 2.5 FPU/g biomass is a low enzyme load, and the combination is between two native and non-concentrated fungal extracts, we consider our results excellent for a first screening of fungal enzymes for 2G ethanol from soy husk.

4 CONCLUSION

Soy husk demonstrated to be a potential raw material for ethanol production, as well as a good carbon source for on-site enzyme production. Additionally, semi-solid media using this biomass, exemplify an interesting alternative to lower the cost of 2G ethanol production. Moreover, the screening process revealed fungi species with great potential to depolymerize soy husk. There is still room for optimization of the selected fungal enzyme cocktails, such as concentration and solid ratio adjustments in the saccharification process, although it has already been demonstrated their ability to release sugars xylose and glucose.

REFERENCES

[1] CLARK, J., DESWARTE, F. (2015). The biorefinery concept: an integrated approach. Introduction to chemicals from biomass, 1-29.
[2] KOUL, B., YAKOOB, M., SHAH, M. P. (2022). Agricultural waste management strategies for environmental sustainability. Environmental Research, 206, 112285.
[3] U. S. Department of Agriculture: Foreign Agricultural Service (2024), Accessed 8 May 2024, <https://fas.usda.gov/data/production/commodity/2222000>.
[4] ADSUL, M., SANDHU, S. K., SINGHANIA, R. R., GUPTA, R., PURI, S. K., MATHUR, A. (2020). Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels. Enzyme and microbial technology, 133, 109442.
[5] JESWANI, H. K., CHILVERS, A., AZAPAGIC, A. Environmental sustainability of biofuels: a review. Proceedings of the Royal Society A, v. 476, n. 2243, p. 20200351, 2020.
[6] CHUKWUMA, O. B., RAFATULLAH, M., TAJARUDIN, H. A., ISMAIL, N. (2020). Lignocellulolytic enzymes in biotechnological and industrial processes: a review. Sustainability, 12(18), 7282.
[7] NIYONZIMA, F. N., VEENA, S. M., MORE, S. S. (2020). Industrial production and optimization of microbial enzymes. Microbial enzymes: roles and applications in industries, 115-135.
[8] GUPTA, A., JANA, A. K. (2019). Production of laccase by repeated batch semi-solid fermentation using wheat straw as substrate and support for fungal growth. Bioprocess and biosystems engineering, 42, 499-512.
[9] SIQUEIRA, J. G. W., RODRIGUES, C., DE SOUZA VANDENBERGHE, L. P., WOICIECHOWSKI, A. L., SOCCOL, C. R. (2020). Current advances in on-site cellulase production and application on lignocellulosic biomass conversion to biofuels: a review. Biomass and Bioenergy, 132, 105419.
[10] NREL Laboratory Analytical Procedure (LAP). Determination of Ash in Biomass. Ohio, 2008. (Technical Report NREL/TP-510-42622).
[11] MILLER, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Analytica Chemistry, 31, 426–430, 1959.
[12] BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.

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