JOÃO BATISTA DE SOUZA

COQUETÉIS ENZIMÁTICOS DE FUNGOS PARA HIDRÓLISE DE CASCA DE SOJA E PRODUÇÃO DE ETANOL

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Magister Scientiae*.

Orientadora: Gabriela Piccolo Maitan-Alfenas

VIÇOSA – MINAS GERAIS

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Gabriela Piccolo Maitan-Alfenas

Orientadora

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RESUMO

SOUZA, João Batista de, M.Sc., Universidade Federal de Viçosa, agosto de 2024. **Coquetéis enzimáticos de fungos para hidrólise de casca de soja e produção de etanol**. Orientadora: Gabriela Piccolo Maitan-Alfenas.

O etanol de segunda geração (2G) é um combustível alternativo que ajuda a acelerar a transição dos combustíveis fósseis para fontes de energia mais sustentáveis, de baixo impacto e economicamente viáveis. Sua produção consiste em quatro etapas principais: pré-tratamento, hidrólise enzimática, fermentação e destilação. Muitos resíduos agrícolas podem ser processados para serem convertidos em etanol 2G. Mais de 300 milhões de toneladas de soja são produzidas em todo o mundo, gerando milhões de toneladas de casca de soja. Este trabalho teve como objetivo produzir enzimas lignocelulósicas *on-site*, utilizando bagaço de cana-de-açúcar, casca de soja e farelo de trigo para o cultivo de *Aspergillus sydowii, Chrysoporthe cubensis, Hypoxylon* sp., *Kretzschmaria zonata* e *Talaromyces pinophilus* por fermentação semissólida. Os coquetéis enzimáticos de fungos foram aplicados para hidrolisar casca de soja após os pré-tratamentos hidrotérmico, ácido (H_2SO_4 0,5 %) e alcalino (NaOH 1,0 %), com foco na liberação de açúcares fermentescíveis para produção de etanol. Após análises de composição química e hidrólise enzimática com o coquetel comercial Cellic® CTec3 HS (Novozymes), o pré-tratamento ácido teve maior impacto na liberação de glicose e xilose, com 13,7 e 7,10 g/L, respectivamente. O perfil enzimático dos 5 fungos cultivados nas diferentes biomassas foi avaliado e destacaram se os extratos de *A. sydowii, C. cubensis* e *T. pinophilus* cultivados em casca de soja, bem como de *C. cubensis* cultivado em farelo de trigo. Quando a etapa de hidrólise enzimática foi realizada em casca de soja pré-tratada com ácido, os extratos de casca de soja *C. cubensis* e *T. pinophilus* demonstraram os melhores resultados, convertendo 9,55 e 13,21 % da celulose em glicose. Porém, quando combinados, nas mesmas condições e carga enzimática (2,5 unidades FPase), a taxa de conversão de celulose em glicose aumentou para 36,35 %, mostrando grande potencial como extratos nativos com baixo uso de enzimas. Além disso, ensaios de fermentação foram conduzidos na casca de soja pré-tratada com ácido, hidrolisada pela mistura dos extratos de *C. cubensis* e *T. pinophilus* crescidos em casca de soja, e *Saccharomyces cerevisiae* Innova® Force ADY foi capaz de se adaptar ao meio complexo e gerar 2,03 g/L de etanol, que representa 66,34 % de conversão da glicose em etanol.

Palavras-chave: *Fungos. Enzimas. Etanol de segunda geração. Casca de soja. Biorrefinarias*.

ABSTRACT

SOUZA, João Batista de, M.Sc., Universidade Federal de Viçosa, agosto de 2024. **Fungal enzymatic cocktails for soy husk hydrolysis and ethanol production**. Adviser: Gabriela Piccolo Maitan-Alfenas.

Second-generation (2G) ethanol is an alternative fuel that helps to accelerate the transition from fossil fuels to more sustainable, low-impact and economically reliable sources of energy. Its production consists of four main steps: pretreatment, enzymatic hydrolysis, fermentation and distillation. Many agricultural wastes can be processed to be converted into 2G ethanol. More than 300 million tons of soybean is produced throughout the world leaving millions of tons of soy husk. This work aimed to produce *on-site* lignocellulosic enzymes, using sugarcane bagasse, soy husk, and wheat bran to grow *Aspergillus sydowii, Chrysoporthe cubensis, Hypoxylon* sp., *Kretzschmaria zonata*, and *Talaromyces pinophilus* by semi-solid fermentation. The fungi enzymatic cocktails were applied to hydrolyze hydrothermal, acid (H_2SO_4 0.5 %) and alkaline (NaOH 1.0 %) pretreated soy husk focusing on fermentable sugars release for ethanol production. After chemical composition analyzes and enzymatic hydrolysis with the commercial cocktail Cellic® CTec3 HS (Novozymes), acid pretreatment had the biggest impact on glucose and xylose release, with 13.7 and 7.10 g/L, respectively. The enzymatic profile of the 5 fungi grown in the different biomasses was evaluated and the extracts of *A. sydowii, C. cubensis* and *T. pinophilus* cultivated in soy husk, as well as *C. cubensis* cultivated in wheat bran outstood. When the enzymatic hydrolysis step was performed on acid-pretreated soy husk, *C. cubensis* and *T. pinophilus* soy husk extracts demonstrated the best results, converting 9.55 and 13.21 % of cellulose to glucose conversion. However, when combined, in the same conditions and enzyme load (2.5 Fpase units), the cellulose to glucose conversion rate increased to 36.35 %, showing great potential as native extracts with low enzyme usage. Furthermore, fermentation assays were conducted on the acid-pretreated soy husk hydrolyzed with the blended *C. cubensis* and *T. pinophilus* soy husk extracts, and *Saccharomyces cerevisiae* Innova® Force ADY was able to adapt to the complex media and generate 2.03 g/L of ethanol, which represents 66.34 % of glucose to ethanol conversion.

Keywords: *Fungi. Enzymes. Second-generation ethanol. Soy husk. Biorefineries.*

SUMÁRIO

INTRODUÇÃO GERAL

O uso de fontes alternativas de energia é uma realidade, visando diminuir a dependência dos combustíveis fósseis, devido aos seus impactos negativos, como acúmulo de gases na atmosfera, mas também, por sua instabilidade econômica, fazendo assim com que a diversificação energética seja foco no planejamento econômico de diversos países (Liu, 2021). Um combustível com grande potencial na transição energética para fontes mais sustentáveis e economicamente viáveis é o etanol, já usado em diversos países. Sua produção é dividida em duas categorias básicas: o etanol de primeira geração (1G) e de segunda geração (2G).

O etanol 1G é produzido pela fermentação, seguida de destilação, do caldo da moagem direta cultivares agrícolas como milho, nos Estados Unidos, cana-de-açúcar, no Brasil e beterraba, na Europa. Este processo é o mais utilizado e com poucas etapas de produção, sendo bem difundido mundialmente. O etanol 2G, por sua vez, pode ser produzido a partir de resíduos agrícolas diversos, como cascas, palhas, sabugo, farelos, bagaços, entre outros. A principal vantagem de seu uso é a matéria prima utilizada, constituída por resíduos descartados das cadeias de produção dos respectivos cultivares, além da utilização praticamente integral do cultivar original (Rabelo, 2019).

A soja é uma cultura com mais de 300 milhões de toneladas produzidas anualmente (USDA, 2024). Brasil e Estados Unidos lideram o ranking mundial, com mais de 100 milhões de toneladas. Após, aparece a Argentina, outro importante produtor, com quase 50 milhões de toneladas (Fig 1). Também vale mencionar países como China, Índia e Paraguai, com produções de aproximadamente 20, 12 e 10 milhões de toneladas, respectivamente (USDA, 2024).

O principal subproduto da sua cadeia de produção é a casca da soja. Cerca de 7 % do peso da soja é proveniente da casca, logo, considerando a produção mundial, mais de 20 milhões de toneladas deste subproduto são geradas anualmente. Este resíduo agrícola é considerado uma biomassa lignocelulósica, constituído principalmente de parede celular, formada pelos polissacarídeos celulose e hemicelulose, assim como a lignina, um polímero aromático extremamente complexo, formado a partir dos álcoois p-cumarílico, coniferílico e sinapílico. Estas três moléculas interligadas fornecem características importantes às plantas, como proteção a impactos e microrganismos, resistência e rigidez (Yousuf, 2020). Devido à complexidade das biomassas lignocelulósica, a produção de etanol 2G é constituída de mais etapas que o etanol 1G, como o pré-tratamento, a hidrólise enzimática ou sacarificação, a fermentação e, por último, a destilação .

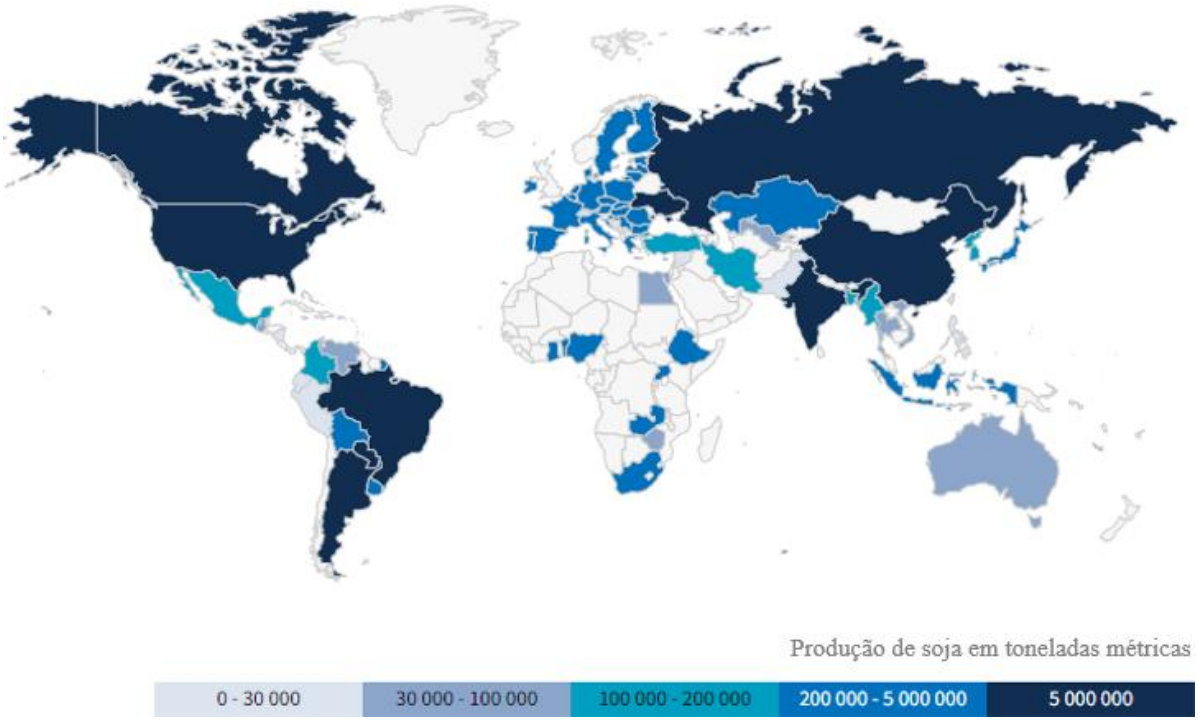

Fig 1 - Produção 2023/2024 de soja no mundo em toneladas. Adaptado de: *U. S. Department of Agriculture: Foreign Agricultural Service*.

O pré-tratamento da biomassa vegetal é a primeira etapa do processo de produção de etanol 2G e visa alterar a estrutura inicial, deixando-a mais acessível às enzimas, seja por ação mecânica (pré-tratamento físico), pelo uso de reagentes (pré-tratamento químico) ou pelo uso de microrganismos e enzimas (pré-tratamento biológico). O pré-tratamento representa, geralmente, mais de 20 % do custo inerente à produção de etanol 2G e interfere diretamente na eficiência das etapas posteriores. Os pré-tratamentos químicos são os mais utilizados, pela alta disponibilidade dos reagentes e um menor custo relativo. Cada tipo de pré-tratamento age de uma forma diferente em cada biomassa específica. Dentre eles, se destacam os pré-tratamentos ácido e alcalino, conhecidos pela despolimerização da hemicelulose e lignina, respectivamente (Woiciechowski, 2020). O pré-tratamento hidrotérmico também é uma alternativa, utilizando água sob alta pressão para causar uma abertura das fibras e consequente despolimerização. Todos os pré-tratamentos podem ser realizados por tempos e com intensidades variadas, gerando diferentes graus de impactos na biomassa, dependendo da finalidade de seu uso (Scapini, 2021).

A sacarificação é uma das etapas mais importantes e é, atualmente, o gargalo na produção de etanol 2G, devido ao alto custo das enzimas aplicadas no processo. Este passo consiste basicamente na conversão dos polímeros de celulose e hemicelulose em seus açúcares

monoméricos, que podem ser posteriormente fermentados. A degradação da celulose (Fig 2) é feita por três conjuntos principais de enzimas: endoglucanases, exoglucanases (ou celobiohidrolases) e β-glicosidases. As endoglucanases são responsáveis por quebrar randomicamente a celulose em porções menores, as celobiohidrolases liberam resíduos de celobiose nas extremidades dos fragmentos e, por fim, as β-glicosidases clivam a celobiose em resíduos de glicose (Adsul, 2020).

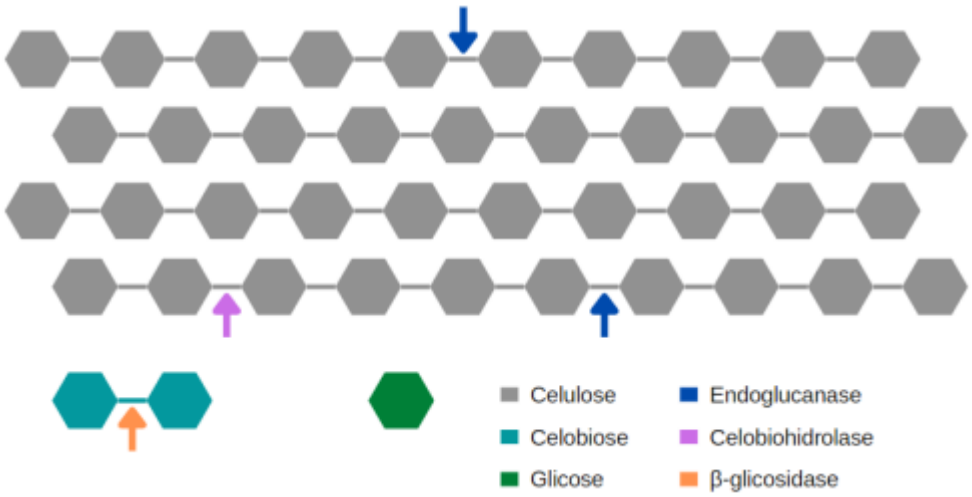

Fig 2 - Principais enzimas envolvidas na degradação da celulose. Adaptado (De Souza, 2013).

A degradação da hemicelulose é mais complexa, devido à sua diversidade estrutural. As hemiceluloses possuem uma variedade de açúcares e outros grupos em sua cadeia e são classificadas, de acordo com esta composição, em xilanas, xiloglucanas, glucomananas e galactomananas, por exemplo (Fig 3). O(s) tipo(s) de hemicelulase(s) assim como a quantidade de cada uma delas varia de acordo com a biomassa vegetal utilizada. A hidrólise das xilanas é feita principalmente pelas β-endoxilanases, que clivam a cadeia randomicamente em oligossacarídeos menores, e β-xilosidases, que quebram estes oligossacarídeos e liberam xilose. As xiloglucanas por sua vez, devido à presença de glicose na sua estrutura, sofrem a ação de endoglucanases e β-glicosidases, assim como a celulose. Já as galacto(gluco)mananas, são clivadas por β-mananases e β-manosidases. No entanto, este conjunto de enzimas hidrolisa apenas a cadeia principal das hemiceluloses, o que torna necessária a ação de diversas enzimas acessórias, para a degradação completa e liberação completa dos açúcares para o meio. Alguns exemplos destas enzimas são α-arabinofuranosidases, α-xilosidases, α-fucosidases, α-galactosidases e feruloil esterases (de Souza, 2013).

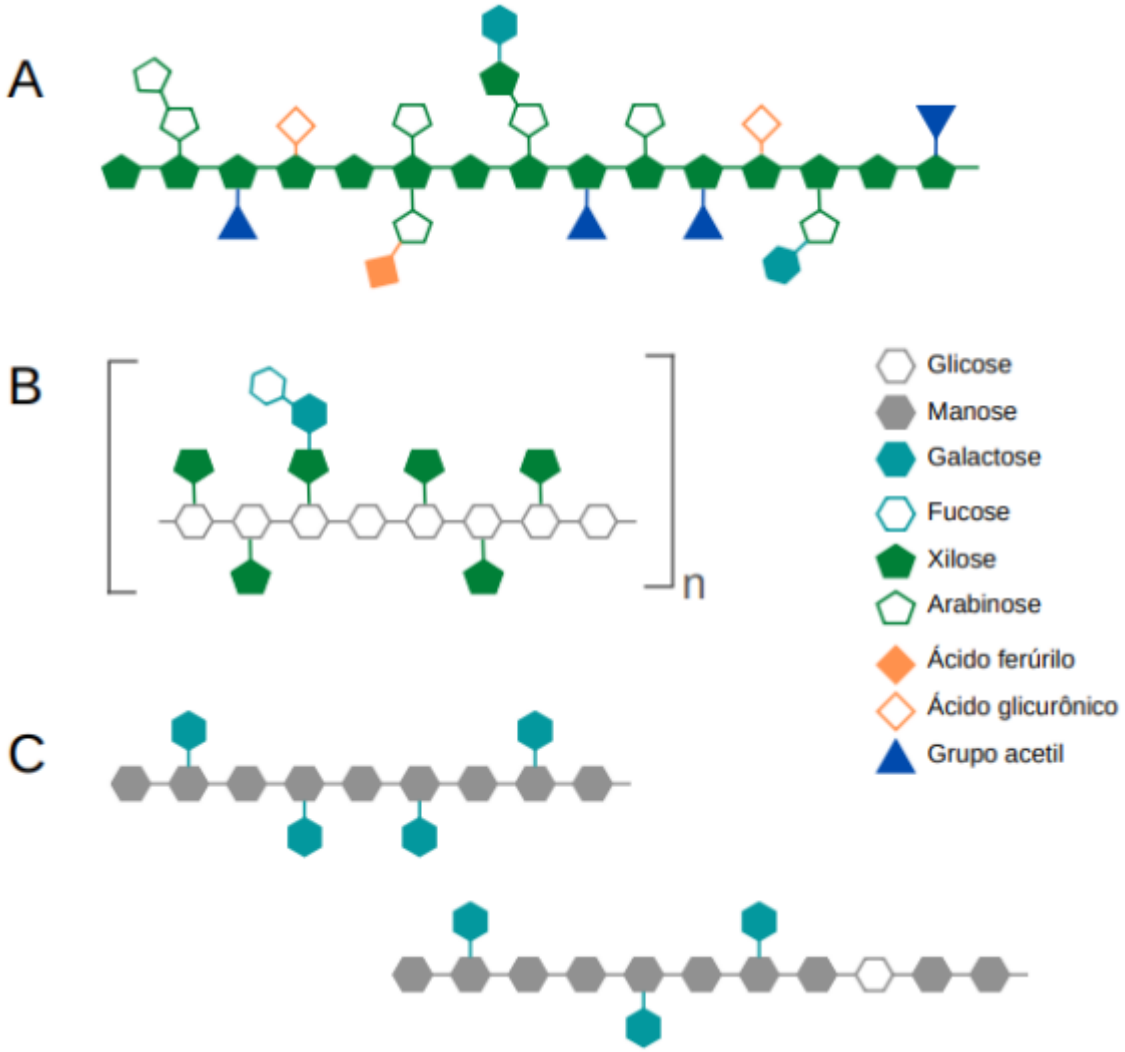

Fig 3 - Esquema estrutural de hemiceluloses. A) Xilana; B) Xiloglucana; C) Galactomanana (esquerda) e Galactoglucomanana (direita). Adaptado: De Souza, 2013.

A fermentação se refere à utilização de microrganismos para produzir o etanol a partir dos açúcares glicose e xilose, como descrito nas equações 1 e 2. Muitos microrganismos são capazes de converter glicose em etanol, com destaque para as leveduras da espécie *Saccharomyces cerevisiae*, que estão estabelecidas e difundidas na produção do combustível (Komesu, 2020). Porém, existem espécies capazes de converter a xilose em etanol, como *Pichia stipitis* e *Spathaspora passalidarum*. O desenvolvimento de cepas capazes de fazer um melhor aproveitamento de todos os açúcares e alcançar o máximo de conversão possível é crucial para diminuir os custos e aumentar os rendimentos da produção de etanol 2G (Hou, 2012).

Hexose

$C_6H_{12}O_6 \leftrightarrow 2C_2H_6O + 2CO_2$ (Equação 1)

Pentose

$3C_5H_{10}O \leftrightarrow 5C_2H_6O + 5CO_2$ (Equação 2)

Por fim, a destilação é o processo que separa e purifica o etanol formado ao longo dos processos anteriores. Esse processo de separação se baseia nos diferentes pontos de ebulição entre os componentes da mistura. Substâncias mais voláteis entram no estado de vapor mais rapidamente, ao se aplicar uma fonte de calor, podendo ser condensadas em um novo recipiente, voltando a forma líquida (Stichlmair, 2021). Ao separar misturas simples, com poucos componentes, este processo é bem direto e consegue alto grau de pureza do componente de interesse. No entanto, na indústria de etanol, sobretudo na de etanol 2G, o meio reacional ao final do processo é bem mais complexo, que necessitam do auxílio de peneiras moleculares e/ou agentes dessecantes para auxiliar no processo de separação (Arlt, 2014).

Dentre as etapas descritas de produção do etanol 2G, a aplicação das enzimas tem o maior custo, sendo o principal gargalo no escalonamento do processo. Logo, estudos visando à produção de enzimas a um baixo custo vêm ganhando notoriedade. Enzimas podem ser produzidas e extraídas de diversos organismos, como plantas, animais, fungos e bactérias. Os microrganismos se destacam, devido a seu fácil cultivo e manutenção (Niyonzima, 2020). Os fungos, além disso, oferecem outras características, como a secreção das enzimas, o que facilita o processo de extração e a possibilidade de serem cultivados em meios semi-sólidos, com uma menor necessidade de água, sendo uma grande vantagem para a indústria. Fungos fitopatogênicos, por exemplo, secretam enzimas capazes de degradar a parede celular vegetal, para infectar os tecidos e absorverem os nutrientes (Alfenas, 2019). Assim, biomassas lignocelulósicas, como a casca de soja, podem ser usadas como fontes de carbono para o cultivo de fungos, para induzir a produção de enzimas específicas para a hidrólise desde material complexo (Adsul, 2020).

A partir destas informações, este trabalho visou produzir coquetéis enzimáticos fúngicos, cultivados em diferentes biomassas, para aplicação na hidrólise da casca de soja, com o intuito de produzir etanol, contribuindo para a redução de custos, facilitando a maior difusão da produção e utilização deste combustível.

OBJETIVOS

Objetivo geral

Produzir e aplicar coquetéis enzimáticos fúngicos para hidrolisar a casca de soja, visando a liberação de açúcares destinados à produção de etanol.

Objetivos específicos

- Avaliar diferentes métodos de pré-tratamento da casca de soja;
- Selecionar fungos que produzam enzimas degradadoras de biomassa lignocelulósica;
- Desenvolver coquetéis enzimáticos fúngicos destinados à hidrólise da casca de soja pré-tratada;
- Fermentar o caldo obtido da hidrólise da casca de soja para obtenção de etanol.

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CAPÍTULO I – Fungal enzymatic mixtures for soy husks saccharification and ethanol production

João Batista de Souza[1], Gabriela Piccolo Maitan-Alfenas[1,] *

[1]Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, 36570-900, Viçosa, MG, Brazil

*Corresponding author: gabriela.maitan@ufv.br; https://orcid.org/0000-0003-2340-0077;

ABSTRACT

Second-generation (2G) ethanol is an alternative fuel that helps to accelerate the transition from fossil fuels to more sustainable, low-impact and economically reliable sources of energy. Its production consists of four main steps: pretreatment, enzymatic hydrolysis, fermentation and distillation. Many agricultural wastes can be processed to be converted into 2G ethanol. More than 300 million tons of soybean is produced throughout the world leaving millions of tons of soy husk. This work aimed to produce *on-site* lignocellulosic enzymes, using sugarcane bagasse, soy husk, and wheat bran to grow *Aspergillus sydowii, Chrysoporthe cubensis, Hypoxylon sp., Kretzschmaria zonata,* and *Talaromyces pinophilus* by semi-solid fermentation. The fungi enzymatic cocktails were applied to hydrolyze hydrothermal, acid (H_2SO_4 0.5 %) and alkaline (NaOH 1.0 %) pretreated soy husk focusing on fermentable sugars releasing for ethanol production. After chemical composition analyzes and enzymatic hydrolysis with the commercial cocktail Cellic® CTec3 HS (Novozymes), acid pretreatment had the biggest impact on glucose and xylose release, with 13.7 and 7.10 g/L, respectively. The enzymatic profile of the 5 fungi grown in the different biomasses was evaluated and the extracts of *A. sydowii, C. cubensis* and *T. pinophilus* cultivated in soy husk, as well as *C. cubensis* cultivated in wheat bran outstood. When the enzymatic hydrolysis step was performed on acid-pretreated soy husk, *C. cubensis* and *T. pinophilus* soy husk extracts demonstrated the best results, converting only 9.55 and 13.21 % of cellulose to glucose conversion. However, when combined, in the same conditions and enzyme load (2.5 Fpase units), the cellulose to glucose conversion rate increased to 36.35 %, showing great potential as native extracts with low enzyme usage. Furthermore, fermentation assays were conducted on the acid-pretreated soy husk hydrolyzed with the blended *C. cubensis* and *T. pinophilus* soy husk extracts, and *Saccharomyces cerevisiae* Innova® Force ADY was able to adapt to the complex media and generate 2.03 g/L of ethanol, which represents 66.34 % of glucose to ethanol conversion.

Keywords: *Fungi. Enzymes. Second-generation ethanol. Soy husk. Biorefineries.*

1 INTRODUCTION

Biorefineries, which convert biomass into energy, materials, and chemicals with higher economic value, are globally increasing, in opposition to the previously dominant fossil fuel industry. They contribute to minimizing environmental impacts and creating more sustainable and economically reliable production chains (Clark, 2015). Second-generation (2G) ethanol for instance, can be produced utilizing a series of agricultural wastes called lignocellulosic biomasses as raw materials, such as straws, bagasse, husks, cobs, and stover (Koul, 2022).

Soybean is a highly cultivated crop, and Brazil, The United States, Argentina, and China lead its production ranks. These four countries produce more than 300 million tons of soybean annually, which generates vast amounts of soy husks, offering a great potential to be converted into 2G ethanol (USDA, 2024). Lignocellulosic biomasses are mainly composed of plant cell wall, a complex structure of the polysaccharides cellulose and hemicellulose intertwined with lignin (Adsul, 2020). Thus, 2G ethanol production is not as straightforward as the first-generation (1G) counterpart. Before fermentation, pretreatment and enzymatic hydrolysis must be performed, to deconstruct the recalcitrant structure of the biomass, releasing fermentable sugars. Cost-effective enzyme production poses the main bottleneck for feasible large-scale 2G ethanol production (Jeswani, 2020).

For cellulose hydrolysis, which is formed by a series of glucose molecules, a small set of enzymes is necessary, mainly endoglucanase, cellobiohydrolase, 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 hemicellulose breakdown (Chukwuma, 2020).

Microorganisms are commonly used as enzyme sources 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 (Niyonzima, 2020). Semi-solid fungi cultivation shows many advantages when applied to biomass conversion since less water is necessary, which is highly important for industries and, moreover, agricultural wastes can be used as carbon sources to induce the production of specific enzymes to depolymerize cellulose and hemicellulose (Gupta, 2019). Hence, soy husk shows a lot of potential to be used as raw material for 2G ethanol production, as well as a carbon source for *on-site* enzyme production, reducing the overall costs of the process (Siqueira, 2020).

This work aimed the production of enzymatic mixtures by different fungi, cultivated in semi-solid media, using wheat bran, sugarcane bagasse, or soy husk as carbon sources, to induce a diverse range of lignocellulolytic enzymes, focusing on pretreated soy husk hydrolysis for releasing sugars destined to ethanol production.

2 MATERIALS AND METHODS

2.1 Soy Husk Pretreatments and Compositional Analyzes

Soy husk was kindly handed out by the CJ Selecta® company. The soy husk was milled to a 20 mesh (0.84 mm) granulometry and t hree pretreatments were performed : hydrothermal, acid H_2SO_4 0.5 % (v/v), and alkaline NaOH 1.0 % (m/v). All treatments were carried with 25 % dry biomass (m/v), at 120 °C for 30 min. Humidity levels were checked with the assistance of an Ohaus MB25 Moisture Analyzer (Cheeselab, São Paulo). After the treatment, the samples were filtered in nylon, extensively washed with distilled water, and dried at 45 °C.

For the compositional analyzes of raw and pretreated samples of soy husk, ash determination was conducted in porcelain crucibles containing 1 g of soy husk. The crucibles were gradually heated until 575 ± 25 °C and kept at this temperature for 3 h. Ash content was estimated by the remaining sample inside the crucible, divided by the initial mass.

Protein content was estimated following the *Kjeldahl* method (Bradstreet, 1954). Initially, 0.3 g of soy husk was digested in a *Kjeldahl* tube with a catalyzing solution composed of $CuSO_4.5H_2O$ + Selenium powder + H_2SO_4 1:1:100 (m:m:v), gradually heated in a digestion block until 340 °C, and maintained for 2h. Then, 1 mL of H_2O_2 was added, and the tube was kept at 340 °C for more 40 minutes. Lastly, 10 mL of distilled water was added to the mixture. The next step was sample distillation in alkaline media, gradually adding NaOH 40 % until the sample showed a brown color. The mixture was subsequently distilled in an Erlenmeyer flask containing 25 mL of H_3BO_3 4 % plus indicator solution. A titration step was carried out, with HCl 0.05 N. The volume of HCl consumed was finally used to calculate the protein content (equation 1).

$$Protein\ (\%) = \frac{V\ x\ f\ x\ N\ x\ 0.0014\ x\ 5.52\ \ x\ 100}{mA} \qquad (1)$$

V = volume of HCl 0.05 N (mL),

f = HCl 0.05 N correction factor,

N = normality of HCl 0.05 N solution and

m(A) = initial sample mass (g).

Extractives content was determined by gravimetry, utilizing 1-2 g of 20 *mesh* (0.84 mm) soy husk inside a filter paper cartridge, within a *Soxhlet* system, with 200 mL of extraction with ethanol 96 °GL and subsequently, distilled water, with 6 h reflux minimum. The percentage of extractives was estimated dividing the weight loss by the total amount of initial dry biomass.

Extractives-free soy husk (0.3 g) went through acid hydrolysis with 3 mL of H_2SO_4 72 %, at 30 °C for 1.5 h. Then, 87 mL of distilled water were added, and the samples were autoclaved at 121 °C for 1 h. The samples were filtered through vacuum in porous glass crucibles. Insoluble lignin was estimated by the solid dry mass retained in the crucibles. Soluble lignin concentration was estimated by spectrophotometry, at 280 nm wavelength, using the filtered hydrolysate (equation 2).

$$A = \varepsilon\, b\, C \qquad (2)$$

ε = molar absorptivity coefficient,

A = absorbance,

b = optical pathway and,

C = concentration.

As proteins also absorb at 280 nm wavelength, the protein content was removed from the total lignin value.

Carbohydrates were estimated by analyzing the acid hydrolysate by *High Performance Liquid Chromatography* - HPLC (CBM-20A/20Alite - Shimadzu). Samples were filtered through 0.45 μm filters. The components were separated by an Aminex HPX-87H column (BioRad) and detected by a refractive index detector (RID-20A – Shimadzu). Twenty μL of the sample were injected into the column and eluted with 0.005 mol/L sulfuric acid solution at a 0.6 mL/min flow, at 65 °C. The peak areas of glucose, xylose, and arabinose were compared to external standards of these sugars injected in the same conditions, at concentrations varying

from 0.5 to 10 g/L. Equations 3 and 4 show how to estimate de cellulose and hemicellulose percentual, respectively.

$$Cellulose\ (\%) = \frac{glucose\ (g)\ x\ 0.9\ x\ 100}{biomass\ weight\ (g)} \quad (3)$$

$$Hemicellulose\ (\%) = \frac{[xylose\ (g) + \ arabinose\ (g)]\ x\ 0.88\ x\ 100}{biomass\ weight\ (g)} \quad (4)$$

2.2 Fungi Culture Conditions and Enzyme Extraction

The fungi *Aspergillus sydowii, Chrysoporthe cubensis, Hypoxylon* sp.*, Kretzschmaria zonata*, and *Talaromyces pinophilus* were kindly donated by the Forest Pathology Laboratory, from the Federal University of Viçosa, and they were maintained in PDA (Potato-Dextrose-Agar) plates. Ten mycelium disks of each fungus were added in semi-solid media with 70 % of moisture (5g of biomass + 12 mL of mineral medium) containing sugarcane bagasse, wheat bran or soy husk as carbon sources. Moreover, mineral medium (NH_4NO_3 1.0 g/L; KH_2PO_4 1.5 g/L; $MgSO_4$ g/L; and $CuSO_4$ 0.25 g/L) plus 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) were added to the media. The flasks were incubated at 28 °C for 8 days. Enzymes extraction was performed 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 Quantification of the Crude Extracts

Different colorimetric methods were used to analyze the activity of 13 different enzymes. Assays based on reducing sugars release were performed with 3,5-dinitrosalicylic acid – DNS (Miller, 1959). Synthetic p-nitrophenyl (pNP) 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 the previously made analytical curves: pNP, with concentrations ranging from 0 to 0.032 µmol, for pNP based assays; and reducing sugars glucose and xylose, with concentrations from 0 to

2.22 µmol, for DNS based assays. For laccase, the molar absorptivity the oxidized ABTS, ε = 3,6 x 10^4 $cm^{-1}.mol^{-1}.L$, was used to calculate its concentration. Spectrometry analysis was performed at different wavelengths for different methods: 540 nm for DNS, 410 nm for *p*NP, 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 was determined with Bradford reagent (Bradford, 1976).

Table 1. Enzymatic profile assays and procedure specifications.

Enzyme	Substrate	Concentration	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 paper (1x6 cm)	-	60	DNS
α-glucosidase	*p*NP-α-D-glucopyranoside	2 mM	15	*p*NP
β-glucosidase	*p*NP-β-D-glucopyranoside	2 mM	15	*p*NP
β-xylosidase	*p*NP-β-D-xylopyranoside	2 mM	15	*p*NP
α-galactosidase	*p*NP-α-D-galactopyranoside	2 mM	15	*p*NP
β-galactosidase	*p*NP-β-D-galactopyranoside	2 mM	15	*p*NP
α-arabinofuranosidase	*p*NP-α-D-arabinofuranoside	2 mM	15	*p*NP
β-cellobiohydrolase	*p*NP-β-D-cellobioside	2 mM	15	*p*NP
Laccase	ABTS	10 mM	15	-

2.4 Enzymatic Hydrolysis of Pretreated Soy Husk

Saccharification assays were carried out using the commercial cocktail Cellic® CTec3 HS (Novozymes), to select the most efficient pretreatment method, following the proportions of 5 % of each pretreated soy husk (m/v), at 20 mesh particle size and an enzyme load of 7 mg of enzyme/g of biomass (2.5 FPU/g) Sodium azide 10 mM and tetracycline 40 µg/mL were added to avoid microbial contamination. The final volume was completed with a citrate buffer 100 mM pH 5.0. The assays were 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 as described in 2.1. Posteriorly, another saccharification assay was performed in the same conditions, using the selected fungal extracts by the enzymatic profile

analyzes. Equations 5 and 6 show how to estimate the cellulose and hemicellulose conversion rate by the glucose and xylose content released by the hydrolysis.

$$Cellulose\ conversion\ (\%) = \frac{glucose\ (g/L)\ x\ V\ x\ 0.9\ x\ 100}{Mb\ x\ cellulose\ content\ (\%)} \quad (5)$$

$$Hemicellulose\ conversion\ (\%) = \frac{xylose\ (g/L)\ x\ V\ x\ 0.88\ x\ 100}{Mb\ x\ hemicellulose\ content\ (\%)} \quad (6)$$

V = Flask total volume (L),
Mb = Initial biomass dry weight (g).

Saccharification assays in the same conditions were performed on the selected pretreated soy husk, using the selected fungal crude extracts with the same 2.5 FPU/g of biomass as well as the blend between the two most efficient extracts, to evaluate a possible cooperation of their different enzymatic arsenals on the biomass depolymerization.

2.5 Fermentation

Fermentation was performed sequentially to the saccharification procedure. All biomass and media content was kept in the flasks, with no fractionation. After the hydrolysis of 1 g acid-pretreated soy husk, as followed in section 2.4, 0.01 g of yeast per g of pretreated biomass were added to the flasks. Three different yeast were used: *Saccharomyces cerevisiae* Innova® Force ADY, *Saccharomyces cerevisiae* CAT-1 and *Spathaspora passalidarum* . The fermentation was carried for 72 hours, at 30 °C and 150 rpm agitation. Aliquots were analyzed at the fermentation times 0, 12, 24, 48 and 72 hours. The aliquots were centrifuged at 16000 *g* for 5 minutes and the supernatant was diluted 3 times for High-Performance Liquid Chromatography analyzes of glucose, xylose, and ethanol. Concentrations of the ethanol external standards varied from 0.5 to 5 g/L. Ethanol conversion can be estimated as followed by equation 7.

$$Ethanol\ conversion(\%) = \frac{ethanol\ (g/L)\ x\ 100}{initial\ glucose\ (g/L)\ x\ 0.51} \quad (7)$$

2.6 Enzymes cooperation assay of the blended extract

The two most efficient crude extracts on pretreated soy husk hydrolysis were analyzed, individually and blended, to compare the expected and real activities of endoglucanase, xylanase, β-glucosidase and β-xylosidase. The proportion of each extract in the blend was established based on FPase activity. This same extract proportion was kept for all assays. The expected activity was estimated by the sum of the individual activities. The enzymatic assays were performed as followed in section 2.3.

2.7 Statistical Analyzes

The statistical analyzes were conducted to compare the data of soy husk chemical compositions, enzyme activities, enzymatic hydrolysis products, and fermentation yields, 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 AND DISCUSSION

3.1 Pretreatments and Compositional Analyzes

The effects on the soy husk chemical composition after each pretreatment is exhibited in Table 2.

Table 2. Chemical composition of the untreated and pretreated soy husk. All components were estimated considering 100 g of initial biomass.

	Untreated	Hydrothermal	Alkaline	Acid
Cellulose (g)	36.47 ± 0.84	34.03 ± 0.51	32.77 ± 0.77	33.08 ± 1.86
Hemicellulose (g)	24.22 ± 0.49	23.25 ± 1.05	18.09 ± 0.48	18.56 ± 1.12
Lignin (g)	10.48 ± 2.28	5.02 ± 1.43	9.82 ± 1.80	7.69 ± 1.78
Protein (g)	14.40 ± 2.46	15.85 ± 1.42	10.35 ± 1.02	15.05 ± 0.93
Ash (g)	4.61 ± 0.25	4.23 ± 0.32	6.64 ± 1.66	2.42 ± 0.21
Extractives (g)	9.94 ± 1.43	11.31 ± 0.88	11.53 ± 1.12	12.04 ± 0.67
Recovery (g)	100.00	96.00	84.84	86.00

Chemical and physical-chemical pretreatments, such as alkaline, acid and hydrothermal, have been extensively used in several agricultural byproducts to disrupt the

lignocellulosic matrix, reducing cellulose crystallinity, opening the compacted and intertwined structure or enabling the cleavage of glucosidic bonds (Lorenci Woiciechowski, 2020). When biomass is pretreated, a portion of its components is solubilized into the liquid fraction. During the washing process, these solubilized components are lost. However, this loss is redressed by the ease of access to the polysaccharides cellulose and hemicellulose in the subsequent steps. Thus, a balance between pretreatment severity and recovery rate needs to be achieved to avoid important material loss. Regarding these aspects, the mild pretreatments tested in this work showed a high recovery rate. Hydrothermal pretreatment maintained 96 % of the initial soy husk dry mass, while NaOH 1.0 % alkaline pretreatment and H_2SO_4 0.5 % acid pretreatment showed 84.84 and 86 % of recovery, respectively.

The use of low concentrations of chemicals is more cost-effective and it was observed that they did not generate saccharification and fermentation inhibitors, such as furfural, hydroxymethylfurfural and phenylic compounds, as commonly seen in higher concentrations and longer times (Jönsson, 2016).

When comparing the overall composition, only slight changes can be perceived for cellulose and hemicellulose fractions. However, the absolute amount of each component does not directly reflect the structural alterations and the accessibility of cellulose and hemicellulose, as well as their impacts in glucose and xylose release. Fig 4 shows the impact on enzymatic hydrolysis of hydrothermal, alkaline, and acid pretreated soy husk by the commercial cocktail Cellic® CTec3 HS.

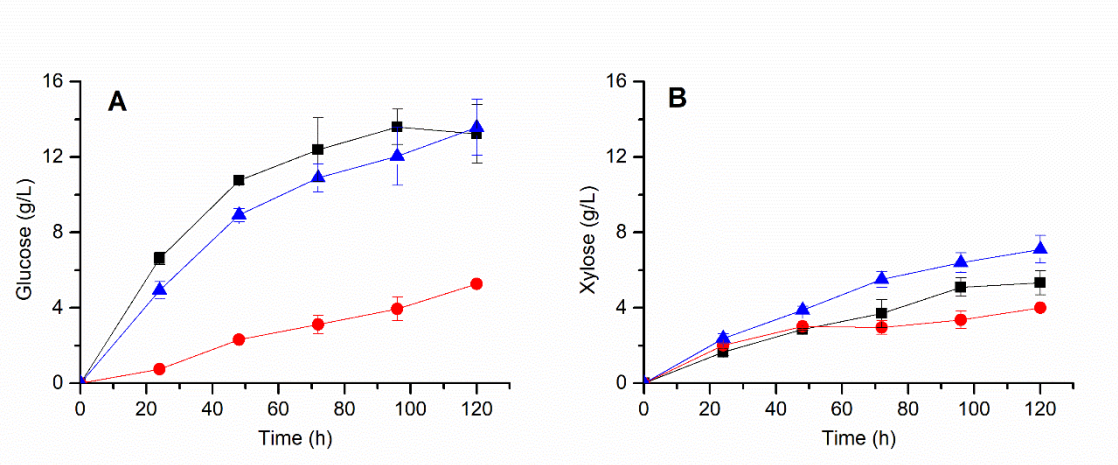

Fig 4 - Glucose (A) and xylose (B) release in the enzymatic hydrolysis of alkaline (■), hydrothermal (●) and acid (▲) pretreated soy husk by the commercial cocktail Cellic® CTec3 HS.

Hydrothermal pretreatment had the smallest effect on glucose release, 5.27 g/L, which represents 26.00 % of cellulose to glucose conversion. Alkaline and acid pretreatments released 13.22 and 13.57 g/L, respectively, both statistically equivalent. Alkaline pretreatment was able to convert 71,93 % of cellulose to glucose and acid pretreatment, 73,84 %. Cellulose depolymerization was not enough to inform whether the alkaline or acid pretreatment was the most efficient. However, when xylose rates were compared, acid pretreatment hydrolysis showed 7.10 g/L, which means 67,.33 % of hemicellulose to xylose conversion, slightly higher than the alkaline, with 5.33 g/L, that is equivalent to 38.74 % of hemicellulose depolymerization. Thus, regarding the glucose plus xylose release throughout the entire process , acid pretreatment was chosen for further testing with the fungal extracts.

3.2 Enzymatic profile and fungi extracts selection

Aspergillus sydowii is a saprotrophic fungus of diverse soil and marine ecosystems that has captured great attention from researchers, due to its production of significant biotechnological metabolites and enzymes (Ibrahim, 2023). Bailin Cong analyzed an *A. sydowii* strain transcriptome and revealed the presence of CAZymes related to lignocellulose degradation (Cong, 2017). In this work, it exhibited high activities for xylanase, 25.99 U/mL, endoglucanase, 1.26 U/mL, mannanase, 1.91 U/mL, and β-glucosidase, 1.66 U/mL, when cultivated in soy husk.

Chrysoporthe cubensis is a plant pathogen that affects mainly Eucalyptus plantations, causing canker disease (Soares, 2018). In order to infect a plant, phytopathogen fungi secrete a series of enzymes to degrade the cell wall, responsible for the host protection. By depolymerizing lignin and the polysaccharides in the cell wall, the fungi have more access to nutrients and can easily spread through the tissues. The secretion capacity is closely related to the fungus pathogenicity (Alfenas, 2019). Tavares, M. P. analyzed the exoproteome of *C. cubensis* cultivated in wheat bran and it produced 26 cellulose degrading enzymes, 39 hemicellulose degrading enzymes and 16 lignin degrading enzymes, besides other enzymes related to chitin, pectin and starch hydrolysis (Tavares, 2024). *C. cubensis* was able to produce a diverse range of enzymes when cultivated in all tested biomasses: sugarcane bagasse, soy husk and wheat bran. However, soy husk stood out for pectinase and β-glucosidase production, with 3.1 and 1.89 U/mL respectively, and wheat bran provided higher activities for endoglucanase and xylanase, with 2.73 and 18.93 U/mL.

Little is known about the enzymatic profile of *Hypoxylon* sp. fungi. Pimentel, D. C. cultivated *Hypoxylon* sp. in pine by solid state fermentation and induced a significant production of mannanase, pectinase, β-galactosidase, β-xylosidase, α-galactosidase, among others (Pimentel, 2024). This fungus was able to produce all these enzymes when grown in wheat bran and soy husk, although in smaller amounts. However, in sugarcane bagasse it produced 2.05 U/mL of pectinase, despite sugarcane bagasse demonstrating a lower protein content than wheat bran and soy husk, which is usually limiting for enzyme production.

Kretzschmaria zonata is reported as the cause of root and collar rot in Teak (*Tectona grandis*), a highly valued wood worldwide (Magaña-Álvarez, 2022). Da Luz Morales published the first report of the enzymatic profile of *K. zonata* grown in a variety of biomasses and showed how the profile changed when cultivated in corn cob, wheat bran, soy husk, teak heartwood, elephant grass and green coffee husk. In teak heartwood, low diversity and low activities were achieved, which is expected for woody materials as less complex biomasses. All the clothes biomasses induced a vast range of hemicellulolytic enzymes, with the highlight for xylanase production in elephant grass extract (da Luz Morales, 2021). In this work, *K. zonata* also exhibited a diverse profile, especially in soy husk, with high activities for pectinase (2.02 U/mL) and α-galactosidase (1.06 U/mL), and significant activity for α-arabinofuranosidase (0.55 U/mL).

Talaromyces pinophilus and other endophytic fungi are gaining interest from the industry due to the production of bioactive compounds (Vinale, 2017). Li, C. X. sequenced the genome of *T. pinophilus*, and 803 genes were annotated as encoding carbohydrate-active enzymes and, from these, 156 were predicted to be related to plant cell wall degradation (Li, 2017). These traits indicate its potential to produce enzymes for application in biomass hydrolysis. *T. pinophilus* stood out when compared to the other fungi in this study, with significant activities for the most tested enzymes when cultivated in wheat bran and soy husk. Soy husk extract was yet the highlight, showing 4.75 U/mL of mannanase, 13.04 U/mL of pectinase, 7.83 U/mL of xylanase, 2.41 U/mL of α-galactosidase and 1.21 U/mL of β-galactosidase.

When growing fungi in solid state fermentation, wheat bran is a widely used low cost carbon source and is known for its outstanding inducing capacity, due to its protein content (Katileviciute, 2019). Nevertheless, soy husk, which is a less common carbon source, still was able to stand out among the tested biomasses. Soy husk demonstrated equivalent or higher activity values for most enzymes and fungi. Besides cellulose and hemicellulose, which are

essential for lignocellulolytic enzymes production, its high protein availability seemed to stimulate fungal development. Thus, these results highlight the potential of soy husk as raw material for high value products conversion, as well as a good carbon source for *on-site* enzyme production. Table 3 summarizes the highest enzymatic activities, after analyzing the replicates with ANOVA and Tukey's test. The detailed enzymatic activities for each crude extract and enzyme tested is showed in section "APÊNDICE I".

Table 3 - Extracts with the highest enzymatic activity values.

Enzyme	Activity (U/mL)	Fungus	Carbon source
Endoglucanase	2.731 ± 0.337	*C. cubensis*	Wheat bran
Mannanase	4.747 ± 0.308	*T. pinophilus*	Soy husk
Pectinase	13.039 ± 0.386	*T. pinophilus*	Soy husk
Xylanase	25.987 ± 1.042	*A. sydowii*	Soy husk
α-Glucosidase	0.036 ± 0.004	*C. cubensis*	Wheat bran
β-Glucosidase	1.889 ± 0.120	*C. cubensis*	Soy husk
β-Xylosidase	0.185 ± 0.008	*T. pinophilus*	Wheat bran
α-Galactosidase	2.411 ± 0.172	*T. pinophilus*	Soy husk
β-Galactosidase	1.215 ± 0.104	*T. pinophilus*	Soy husk
α-Arabinofuranosidase	0.655 ± 0.030	*C. cubensis*	Soy husk
β-Cellobiohydrolase	0.353 ± 0.044	*C. cubensis*	Soy husk
Laccase	0.040 ± 0.004	*C. cubensis*	Soy husk

T. pinophilus and *C. cubensis* cultivated in soy husk noticeably surpassed the other extracts in most of the tested enzymes, with a diverse enzymatic arsenal. *C. cubensis* cultivated in wheat bran was the next best extract and the extract of *A. sydowii* in soy husk exhibited a high xylanase activity for a crude extract, a very important enzyme for hemicellulose degradation. Therefore, the enzymatic extracts of *T. pinophilus*, *C. cubensis* and *A. sydowii* cultivated in soy husk, and *C. cubensis* cultivated in wheat bran were used for further testing in the depolymerization of acid pretreated soy husk.

3.3 Enzymatic hydrolysis of pretreated soy husk by fungi crude extracts

All four selected extracts were able to hydrolyze the acid-pretreated soy husk to some extent. Fig 5 compares the glucose and xylose release throughout the saccharification process.

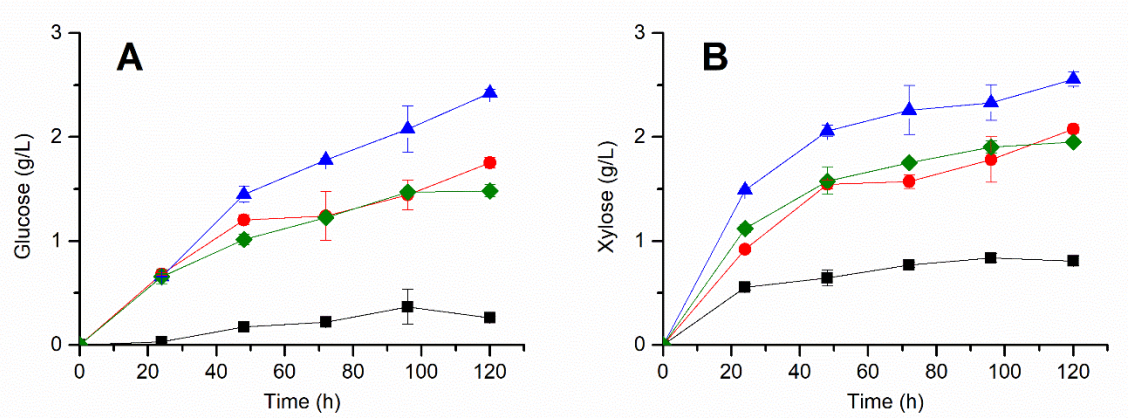

Fig 5 - Glucose (A) and xylose (B) released in acid-pretreated soy husk hydrolyzed with the four selected enzymatic extracts: *A. sydowii* cultivated in soy husk (■), *C. cubensis* cultivated in soy husk (●), *C. cubensis* cultivated in wheat bran (◆) and *T. pinophilus* cultivated in soy husk (▲).

Despite the outstanding xylanase activity of the *A. sydowii* extract, its hydrolytic power was low, generating only 0.26 and 0.82 g/L of glucose and xylose, respectively. This extract may show great potential for xylanase production, purification and application of this enzyme in many fields. However, for greater monosaccharides conversion, the enzymatic hydrolysis process needs the conjunct action of glycoside hydrolases and auxiliary activity enzymes to better degrade cellulose and hemicellulose, as discussed by Maitan-Alfenas (Maitan-Alfenas, 2015). Both extracts of *C. cubensis* had a similar effect on acid-pretreated soy husk saccharification. The soy husk extract was slightly better, with 1.75 and 2.08 g/L of glucose and xylose production, respectively, surpassing the wheat bran counterpart, with 1.48 and 1.95 g/L of glucose and xylose, respectively, after 5 days of hydrolysis.

Dutra, T. R. demonstrated the capacity of hydrolysis of a *C. cubensis* extract cultivated in sugarcane bagasse under solid state fermentation, releasing 2.30 g/L of glucose and 3.91 g/L of xylose, after 3 days of hydrolysis using 10 FPase units per gram of biomass, in pretreated sugarcane bagasse saccharification (Dutra, 2017). For soy husk hydrolysis, fewer studies are available and this biomass is more complex when compared to sugarcane bagasse. In this work, both *C. cubensis* extracts were able to produce similar amounts of glucose and xylose, hydrolyzing soy husk for 5 days, however, using 2.5 FPase units per gram of pretreated biomass, one quarter of the enzyme load of the previous study, making the process more cost-effective, regarding the high costs of enzymes in the market.

Li, C. X. analyzed the genome of *T. pinophilus* to show its enzymes potential for industrial applications. It was found a great number of CAZymes, such as glycoside hydrolases,

with 72 families, 5 families of glycosyl transferases, 13 families of carbohydrate esterases, 10 families of auxiliary activities among others, showing the possible applications of this fungus in biomass conversion (Li, 2017). In this work, its potential was confirmed, since *T. pinophilus* extract cultivated in soy husk stood out among the selected crude extracts, with 2.42 g/L of glucose and 2.55 of xylose release.

De Oliveira Rodrigues, P. evaluated a consortium of A*spergillus fumigatus* and *Aspergillus niger* extracts in the enzymatic hydrolysis of sugarcane bagasse. The mixed extract was able to cause the highest reducing sugars production, after 12 hours of hydrolysis, with 38.22 % of conversion rate (de Oliveira Rodrigues, 2017). In this work, the extracts of *C. cubensis* and *T. pinophilus*, that stood out in the acid-pretreated soy husk hydrolysis were evaluated together, to verify whether their diverse enzyme arsenal would complement each other or not. Fig 6 shows the glucose and xylose released by the extracts tested individually and combined.

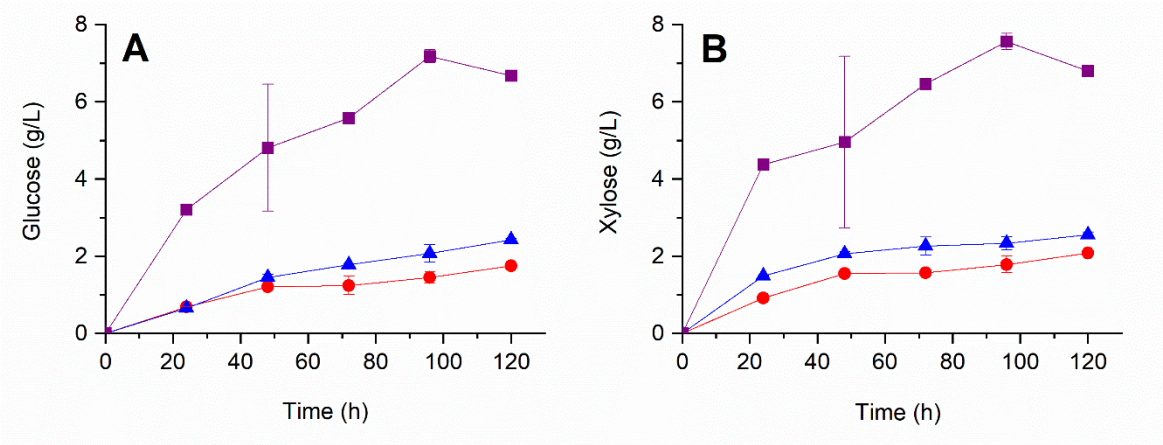

Fig 6 - Glucose (A) and xylose (B) released after acid-pretreated soy husk saccharification by the blended extract (■) compared to the individual extracts of *C. cubensis* (●) and *T. pinophilus* (▲) cultivated in soy husk.

Initially, *C. cubensis* and *T. pinophilus* extracts released 1.72 and 2.42 g/L of glucose, representing 9.55 and 13.21 % of total cellulose conversion, respectively. The blended extract highly increased glucose production up to 6.67 g/L . The released glucose was equivalent to 36.35 % of conversion. When xylose content was evaluated, *C. cubensis* and *T. pinophilus* extracts released 2.08 and 2.55, which correspond to hemicellulose conversion rates of 19.72 and 24.18 %, respectively. The xylose release caused by the blended extract was 6.79 g/L, or 64.39 % of hemicellulose conversion. This represents a significant impact on the hydrolysis

capacity. Compared to the individual counterparts, the sugars release was more than doubled, without increasing the total FPase units of the enzyme mixture, suggesting a strong cooperation between the enzymes of the extracts.

3.4 Fermentation

The first microorganism that comes to mind when alcoholic fermentation is involved is *Saccharomyces cerevisiae*. It is the best-studied eukaryote and a valuable tool for research and several industrial applications, and it is, consequently, the most established yeast in ethanol production, due to its high product tolerance and media adaptability (Parapouli, 2020). For first-generation (1G) ethanol production, the fermentation process is simpler and more straightforward, since the sugar content is high, and the yeast osmotic stress is lower. However, for the 2G ethanol production, a more complex media is obtained, with less sugar available, and high solid rates, interfering in yeast growth, because of low access to nutrients and substrate for action (Nguyen, 2017). For sugarcane and corn, many studies and processes are already industrially implemented. However, for soybean waste materials less information is available. Cortivo, P. R. D. fermented acid and alkaline pretreated soybean hull using *S. cerevisiae*, with ethanol production ranging from 2.3 to 19.8 g/L, consuming all available glucose content (Cortivo, 2018). In this study, acid-pretreated soy husk, hydrolyzed with a low enzyme load (2.5 FPase units/g of biomass) was successfully fermented by the commercial *S. cerevisiae* Innova® Force ADY. Fig 7 illustrates the sugar use and ethanol production over time.

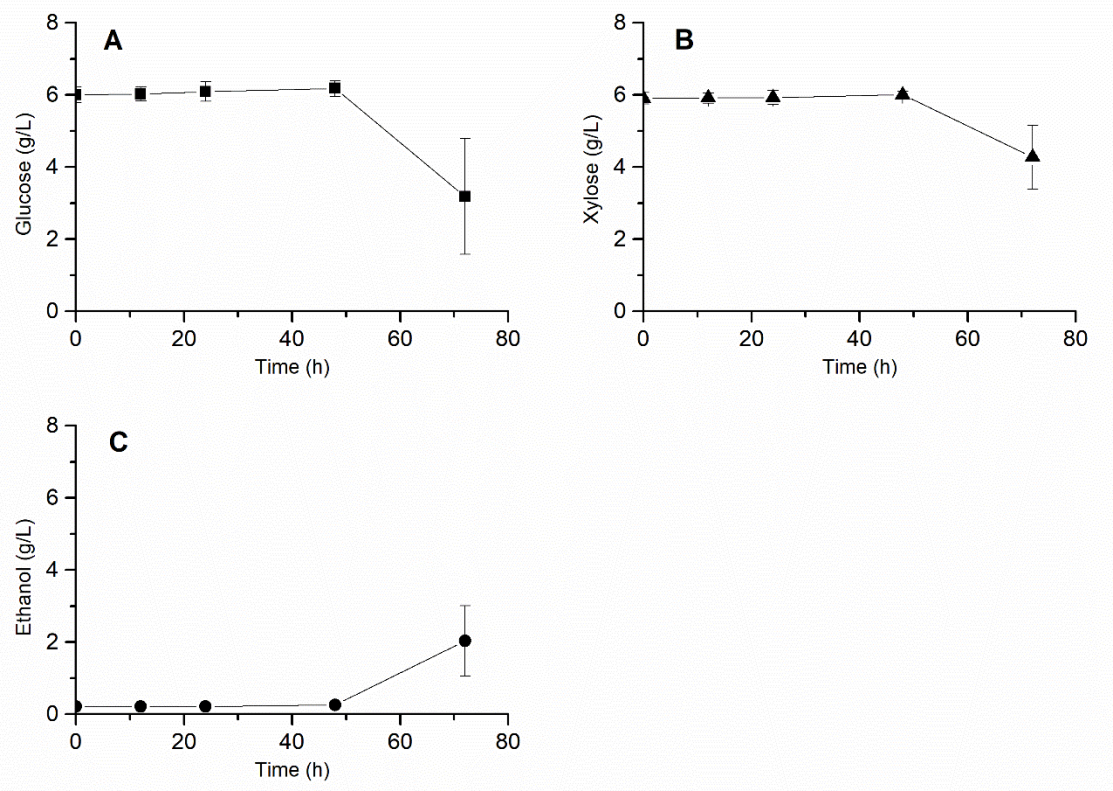

Fig 7 - Glucose (A) and xylose (B) consumption, and ethanol (C) production by *S. cerevisiae* Innova® Force ADY during the 72 hours of fermentation.

As shown by Da Silva Fernandes, F., for lignocellulosic materials fermentation, many challenges need to be overcome by the yeast, such as media complexity, stress, ethanol tolerance. Besides, not all sugars available for the microorganism can be converted into ethanol (da Silva Fernandes, 2022). In this work, even with a 5 % solid rate and a low initial sugar content, when compared to the first-generation process, *S. cerevisiae* Innova® Force ADY was able to adapt and grow using glucose and xylose from the media. Glucose content decreased from 6.00 to 3.18 g/L and xylose from 5.91 to 4.27 g/L. After 72 hours of fermentation, 2.03 g/L of ethanol was produced. If all the initial glucose of the acid-pretreated soy husk was converted into ethanol after the fermentation processes, 0.0612 g would be the total mass of ethanol in each flask. The final concentration of ethanol generated was equivalent 0.0406 g, considering the assay's total volume. That is equivalent to 66.34 % of ethanol yield. *S. cerevisiae* CAT-1 and *S. passalidarum* were not able to generate ethanol in the tested conditions and time. However, *S. cerevisiae* Innova® Force ADY demonstrated significant efficiency, considering the media's harsh conditions.

3.5 Processes overview and perspectives

The effects of the pretreatment methods are not so visible at first glimpse, because the recovery rate is not directly related to the degree of depolymerization of cellulose and hemicellulose. However, the posterior saccharification step confirmed their impacts in sugars release and the comparison highlighted the mild acid pretreatment as the most efficient. One alternative to increase the overall yield is to remove the washing process, maintaining the components depolymerized in this process, given that no inhibitors (such as furfural and hydroxymethylfurfural) were detected.

Saccharification with commercial cocktail Cellic® CTec3 HS demonstrated conversion rates of 66.98 % of cellulose to glucose and 67.33 % of hemicellulose to xylose, showing a lacuna for the depolymerization of soy husk. When the selected extracts were tested in the hydrolysis of acid pretreated soy husk, the blended extract of *T. pinophilus* and *C. cubensis* cultivated in soy husk exhibited the greatest effect, with 36.35 % of cellulose to glucose and 64.39 % of hemicellulose to xylose conversion rates, respectively. When we compare the cellulose hydrolysis, the commercial cocktail is visibly superior to the crude blended extract, which is expected, given that the crude blend was not made in optimized conditions and has no stabilizers to maintain the enzymatic activities at maximum rates overtime. Nonetheless, when we compare the hemicellulose depolymerization, the conversion rate is extremely similar, proving the potential of the fungal enzymatic mixture that can be manufactured with the aid of *T.pinophilus* and *C. cubensis* cultivated in soy husk. Overall, saccharification is still the most challenging step and needs more optimization to guarantee the economic viability of the process.

In the fermentation step, 66.34 % of the initial glucose was converted to ethanol, which is a high conversion rate, regarding the complexity of the media, composed of sugars diluted with the solid content of the biomass, making it a harsh environment for yeast growth. Yet, not all glucose was consumed throughout the fermentation time evaluated. Thus, fermenting for a slightly higher period of time could be an alternative to benefit *S. cerevisiae* development and to increase the ethanol yield.

4 CONCLUSIONS

The mild tested pretreatments, despite the little change in the chemical composition of soy husk, had an impact on the xylose and glucose release during the saccharification process, decreasing the reagent's cost and the impacts after their use. Furthermore, two fungal cocktails

produced on-site, from *T. pinophilus* and *C. cubensis* cultivated in soy husk, were able to successfully hydrolyze the acid pretreated soy husk and demonstrated a significant increase in hydrolytic power when mixed, which suggests that their diverse enzymes arsenal complement each other, showing high synergism. This highlights again, the potential of soy husk for raw material to ethanol conversion, as well as a great carbon source for cell wall degrading enzymes production. Despite the challenges in fermentation of complex media and osmotic stress, the commercial *S. cerevisiae* Innova® Force ADY adapted to the harsh conditions and generated ethanol. This is an initial study of a low-cost and low-impact 2G ethanol production process, successfully performed with the best extracts selected in the screening process. There is still room for optimization and evaluating other enzyme loadings, longer times of fermentation and different solid ratios for hydrolysis and fermentation experiments.

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APÊNDICE A – Perfil enzimático dos fungos crescidos em diferentes biomassas.

Tabela 4 – Atividades de endoglucanase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	Endoglucanase (U/mL)
A. sydowii	bagaço de cana	0,070 ± 0,026 **F**
A. sydowii	casca de soja	1,263 ± 0,073 **C**
A. sydowii	farelo de trigo	0,450 ± 0,125 **DE**
C. cubensis	bagaço de cana	0,445 ± 0,102 **DE**
C. cubensis	casca de soja	1,850 ± 0,166 **B**
C. cubensis	farelo de trigo	2,731 ± 0,337 **A**
Hypoxylon sp.	bagaço de cana	0,253 ± 0,107 **EF**
Hypoxylon sp.	casca de soja	0,227 ± 0,165 **EF**
Hypoxylon sp.	farelo de trigo	0,162 ± 0,153 **EF**
K. zonata	bagaço de cana	0,162 ± 0,153 **DEF**
K. zonata	casca de soja	0,164 ± 0,047 **EF**
K. zonata	farelo de trigo	0,580 ± 0,180 **D**
T. pinophilus	bagaço de cana	0,069 ± 0,059 **F**
T. pinophilus	casca de soja	0,321 ± 0,196 **DEF**
T. pinophilus	farelo de trigo	0,418 ± 0,163 **DE**

Tabela 5 – Atividades de mananase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	Mananase (U/mL)
A. sydowii	bagaço de cana	1,991 ± 0,255 **B**
A. sydowii	casca de soja	1,915 ± 0,167 **B**
A. sydowii	farelo de trigo	0,488 ± 0,078 **EF**
C. cubensis	bagaço de cana	0,366 ± 0,288 **EFG**
C. cubensis	casca de soja	0,910 ± 0,093 **CD**
C. cubensis	farelo de trigo	0,848 ± 0,181 **D**
Hypoxylon sp.	bagaço de cana	0,691 ± 0,148 **DE**
Hypoxylon sp.	casca de soja	1,027 ± 0,541 **C**
Hypoxylon sp.	farelo de trigo	0,729 ± 0,409 **CD**
K. zonata	bagaço de cana	0,255 ± 0,091 **FG**
K. zonata	casca de soja	0,300 ± 0,111 **FG**
K. zonata	farelo de trigo	0,471 ± 0,154 **EF**
T. pinophilus	bagaço de cana	0,041 ± 0,019 **G**
T. pinophilus	casca de soja	4,747 ± 0,308 **A**
T. pinophilus	farelo de trigo	0,976 ± 0,123 **CD**

Tabela 6 – Atividades de pectinase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	Pectinase (U/mL)
A. sydowii	bagaço de cana	0,455 ± 0,045 ^{G}
A. sydowii	casca de soja	0,479 ± 0,030 ^{G}
A. sydowii	farelo de trigo	0,450 ± 0,081 ^{G}
C. cubensis	bagaço de cana	0,647 ± 0,061 ^{EFG}
C. cubensis	casca de soja	3,132 ± 0,160 ^{C}
C. cubensis	farelo de trigo	1,076 ± 0,156 ^{E}
Hypoxylon sp.	bagaço de cana	2,053 ± 0,555 ^{D}
Hypoxylon sp.	casca de soja	0,615 ± 0,105 ^{FG}
Hypoxylon sp.	farelo de trigo	0,759 ± 0,177 ^{EFG}
K. zonata	bagaço de cana	0,823 ± 0,093 ^{EFG}
K. zonata	casca de soja	2,023 ± 0,180 ^{D}
K. zonata	farelo de trigo	1,055 ± 0,289 ^{EF}
T. pinophilus	bagaço de cana	0,627 ± 0,128 ^{FG}
T. pinophilus	casca de soja	13,039 ± 0,386 ^{A}
T. pinophilus	farelo de trigo	3,721 ± 0,178 ^{B}

Tabela 7 – Atividades de xilanase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	Xilanase (U/mL)
A. sydowii	bagaço de cana	1,030 ± 0,178 ^{F}
A. sydowii	casca de soja	25,987 ± 1,042 ^{A}
A. sydowii	farelo de trigo	5,861 ± 0,552 ^{D}
C. cubensis	bagaço de cana	0,693 ± 0,480 ^{F}
C. cubensis	casca de soja	9,133 ± 0,682 ^{C}
C. cubensis	farelo de trigo	18,928 ± 2,570 ^{B}
Hypoxylon sp.	bagaço de cana	0,971 ± 0,378 ^{F}
Hypoxylon sp.	casca de soja	1,645 ± 0,928 ^{F}
Hypoxylon sp.	farelo de trigo	0,663 ± 0,734 ^{F}
K. zonata	bagaço de cana	0,542 ± 0,147 ^{F}
K. zonata	casca de soja	0,733 ± 0,331 ^{F}
K. zonata	farelo de trigo	1,346 ± 0,438 ^{F}
T. pinophilus	bagaço de cana	0,489 ± 0,435 ^{F}
T. pinophilus	casca de soja	7,833 ± 0,413 ^{C}
T. pinophilus	farelo de trigo	3,999 ± 0,409 ^{E}

Tabela 8 – Atividades de FPase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	FPase (U/mL)
A. sydowii	bagaço de cana	0,060 ± 0,021 C
A. sydowii	casca de soja	0,139 ± 0,058 BC
A. sydowii	farelo de trigo	0,088 ± 0,036 C
C. cubensis	bagaço de cana	0,360 ± 0,063 AB
C. cubensis	casca de soja	0,189 ± 0,034 BC
C. cubensis	farelo de trigo	0,202 ± 0,047 BC
Hypoxylon sp.	bagaço de cana	0,351 ± 0,176 AB
Hypoxylon sp.	casca de soja	0,198 ± 0,095 BC
Hypoxylon sp.	farelo de trigo	0,508 ± 0,187 A
K. zonata	bagaço de cana	0,511 ± 0,249 A
K. zonata	casca de soja	0,097 ± 0,015 C
K. zonata	farelo de trigo	0,449 ± 0,177 A
T. pinophilus	bagaço de cana	0,082 ± 0,033 C
T. pinophilus	casca de soja	0,147 ± 0,055 BC
T. pinophilus	farelo de trigo	0,171 ± 0,090 BC

Tabela 9 – Atividades de α-glicosidase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	α-Glicosidase (U/mL)
A. sydowii	bagaço de cana	ND
A. sydowii	casca de soja	ND
A. sydowii	farelo de trigo	0,004 ± 0,002 E
C. cubensis	bagaço de cana	ND
C. cubensis	casca de soja	0,021 ± 0,001 B
C. cubensis	farelo de trigo	0,036 ± 0,004 A
Hypoxylon sp.	bagaço de cana	0,008 ± 0,002 D
Hypoxylon sp.	casca de soja	ND
Hypoxylon sp.	farelo de trigo	ND
K. zonata	bagaço de cana	ND
K. zonata	casca de soja	ND
K. zonata	farelo de trigo	ND
T. pinophilus	bagaço de cana	ND
T. pinophilus	casca de soja	0,003 ± 0,001 E
T. pinophilus	farelo de trigo	0,017 ± 0,001 C

Tabela 10 – Atividades de β-glicosidase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	β-Glicosidase (U/mL)
A. sydowii	bagaço de cana	0,139 ± 0,008 FGH
A. sydowii	casca de soja	1,661 ± 0,100 B
A. sydowii	farelo de trigo	1,152 ± 0,109 C
C. cubensis	bagaço de cana	0,096 ± 0,008 GH
C. cubensis	casca de soja	1,889 ± 0,120 A
C. cubensis	farelo de trigo	1,655 ± 0,077 B
Hypoxylon sp.	bagaço de cana	0,363 ± 0,174 E
Hypoxylon sp.	casca de soja	0,837 ± 0,114 D
Hypoxylon sp.	farelo de trigo	0,290 ± 0,041 EF
K. zonata	bagaço de cana	0,021 ± 0,002 H
K. zonata	casca de soja	0,210 ± 0,053 EFG
K. zonata	farelo de trigo	0,289 ± 0,022 EF
T. pinophilus	bagaço de cana	0,026 ± 0,004 H
T. pinophilus	casca de soja	0,326 ± 0,017 E
T. pinophilus	farelo de trigo	0,958 ± 0,048 D

Tabela 11 – Atividades de β-xilosidase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	β-Xilosidase (U/mL)
A. sydowii	bagaço de cana	0,008 ± 0,001 GHI
A. sydowii	casca de soja	0,021 ± 0,004 E
A. sydowii	farelo de trigo	0,017 ± 0,009 EF
C. cubensis	bagaço de cana	ND
C. cubensis	casca de soja	0,101 ± 0,003 D
C. cubensis	farelo de trigo	0,133 ± 0,006 C
Hypoxylon sp.	bagaço de cana	0,009 ± 0,003 FGH
Hypoxylon sp.	casca de soja	0,019 ± 0,002 E
Hypoxylon sp.	farelo de trigo	0,014 ± 0,005 EFG
K. zonata	bagaço de cana	ND
K. zonata	casca de soja	0,022 ± 0,004 E
K. zonata	farelo de trigo	0,007 ± 0,002 GH
T. pinophilus	bagaço de cana	0,004 ± 0,001 H
T. pinophilus	casca de soja	0,151 ± 0,002 B
T. pinophilus	farelo de trigo	0,185 ± 0,008 A

Tabela 12 – Atividades de α-galactosidase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	α-Galactosidase (U/mL)
A. sydowii	bagaço de cana	0,008 ± 0,003 ^{G}
A. sydowii	casca de soja	0,469 ± 0,025 ^{D}
A. sydowii	farelo de trigo	0,719 ± 0,035 ^{C}
C. cubensis	bagaço de cana	0,007 ± 0,001 ^{G}
C. cubensis	casca de soja	0,179 ± 0,007 ^{EF}
C. cubensis	farelo de trigo	0,150 ± 0,038 ^{EFG}
Hypoxylon sp.	bagaço de cana	0,017 ± 0,002 ^{G}
Hypoxylon sp.	casca de soja	0,201 ± 0,011 ^{E}
Hypoxylon sp.	farelo de trigo	0,044 ± 0,028 ^{EFG}
K. zonata	bagaço de cana	0,029 ± 0,006 ^{FG}
K. zonata	casca de soja	1,063 ± 0,180 ^{B}
K. zonata	farelo de trigo	0,460 ± 0,131 ^{D}
T. pinophilus	bagaço de cana	0,041 ± 0,006 ^{FG}
T. pinophilus	casca de soja	2,411 ± 0,172 ^{B}
T. pinophilus	farelo de trigo	0,991 ± 0,068 ^{A}

Tabela 13 – Atividades de β-galactosidase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	β-Galactosidase (U/mL)
A. sydowii	bagaço de cana	ND
A. sydowii	casca de soja	0,005 ± 0,002 ^{E}
A. sydowii	farelo de trigo	ND
C. cubensis	bagaço de cana	0,007 ± 0,001 ^{E}
C. cubensis	casca de soja	0,077 ± 0,004 ^{D}
C. cubensis	farelo de trigo	0,025 ± 0,001 ^{DE}
Hypoxylon sp.	bagaço de cana	0,020 ± 0,002 ^{E}
Hypoxylon sp.	casca de soja	0,164 ± 0,012 ^{C}
Hypoxylon sp.	farelo de trigo	0,031 ± 0,004 ^{DE}
K. zonata	bagaço de cana	ND
K. zonata	casca de soja	0,032 ± 0,006 ^{DE}
K. zonata	farelo de trigo	0,195 ± 0,025 ^{C}
T. pinophilus	bagaço de cana	0,042 ± 0,009 ^{DE}
T. pinophilus	casca de soja	1,215 ± 0,104 ^{A}
T. pinophilus	farelo de trigo	0,614 ± 0,024 ^{B}

Tabela 14 – Atividades de α-arabinofuranosidase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	α-Arabinofuranosidase (U/mL)
A. sydowii	bagaço de cana	ND
A. sydowii	casca de soja	0,005 ± 0,001 ^{G}
A. sydowii	farelo de trigo	ND
C. cubensis	bagaço de cana	ND
C. cubensis	casca de soja	0,655 ± 0,030 ^{A}
C. cubensis	farelo de trigo	0,227 ± 0,014 ^{C}
Hypoxylon sp.	bagaço de cana	0,032 ± 0,004 ^{FG}
Hypoxylon sp.	casca de soja	0,095 ± 0,006 ^{E}
Hypoxylon sp.	farelo de trigo	0,060 ± 0,024 ^{EF}
K. zonata	bagaço de cana	0,003 ± 0,001 ^{G}
K. zonata	casca de soja	0,547 ± 0,067 ^{B}
K. zonata	farelo de trigo	0,214 ± 0,007 ^{C}
T. pinophilus	bagaço de cana	ND
T. pinophilus	casca de soja	0,163 ± 0,011 ^{D}
T. pinophilus	farelo de trigo	0,011 ± 0,001 ^{G}

Tabela 15 – Atividades de β-celobiohidrolase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	β-Celobiohidrolase (U/mL)
A. sydowii	bagaço de cana	0,020 ± 0,006 ^{GH}
A. sydowii	casca de soja	0,109 ± 0,021 ^{D}
A. sydowii	farelo de trigo	0,198 ± 0,008 ^{C}
C. cubensis	bagaço de cana	0,037 ± 0,004 ^{FG}
C. cubensis	casca de soja	0,353 ± 0,044 ^{A}
C. cubensis	farelo de trigo	0,287 ± 0,020 ^{B}
Hypoxylon sp.	bagaço de cana	0,005 ± 0,003 ^{H}
Hypoxylon sp.	casca de soja	0,090 ± 0,015 ^{DE}
Hypoxylon sp.	farelo de trigo	0,006 ± 0,001 ^{H}
K. zonata	bagaço de cana	ND
K. zonata	casca de soja	0,006 ± 0,004 ^{GH}
K. zonata	farelo de trigo	0,003 ± 0,002 ^{H}
T. pinophilus	bagaço de cana	ND
T. pinophilus	casca de soja	0,008 ± 0,005 ^{GH}
T. pinophilus	farelo de trigo	0,065 ± 0,009 ^{EF}

Tabela 16 – Atividades de lacase dos extratos fúngicos produzidos.

Fungo	Fonte de carbono	Lacase (U/mL)
A. sydowii	bagaço de cana	ND
A. sydowii	casca de soja	ND
A. sydowii	farelo de trigo	ND
C. cubensis	bagaço de cana	ND
C. cubensis	casca de soja	0,040 ± 0,004 [A]
C. cubensis	farelo de trigo	0,023 ± 0,004 [B]
Hypoxylon sp.	bagaço de cana	ND
Hypoxylon sp.	casca de soja	ND
Hypoxylon sp.	farelo de trigo	0,005 ± 0,001 [C]
K. zonata	bagaço de cana	ND
K. zonata	casca de soja	ND
K. zonata	farelo de trigo	0,020 ± 0,009 [B]
T. pinophilus	bagaço de cana	ND
T. pinophilus	casca de soja	ND
T. pinophilus	farelo de trigo	ND

APÊNDICE B – Dados de consumo de açúcares e geração de etanol dos diferentes testes de fermentação.

TESTE 1 – Fermentação com fracionamento, separando o caldo do resíduo lignocelulósico.

Tabela 17 – Consumo de açúcares e geração de etanol usando 0,02 g de células de *Saccharomyces cerevisiae* Innova® Force ADY.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	3,222 ± 0,200	3,580 ± 0,241	ND
24	2,057 ± 0,031	2,278 ± 0,043	ND
48	2,129 ± 0,019	2,213 ± 0,019	ND
60	2,112 ± 0,005	2,197 ± 0,016	ND

Tabela 18 – Consumo de açúcares e geração de etanol usando 0,01 g de células de *Saccharomyces cerevisiae* Innova® Force ADY.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	3,167 ± 0,414	3,503 ± 0,491	ND
24	1,962 ± 0,116	2,111 ± 0,141	ND
48	1,962 ± 0,034	2,086 ± 0,040	ND
60	2,006 ± 0,038	2,138 ± 0,057	ND

Tabela 19 – Consumo de açúcares e geração de etanol usando 0,02 g de células de *Saccharomyces cerevisiae* CAT-1.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	3,434 ± 0,363	3,825 ± 0,392	ND
24	2,300 ± 0,082	2,409 ± 0,097	ND
48	2,082 ± 0,036	2,139 ± 0,038	ND
60	2,138 ± 0,007	2,194 ± 0,003	ND

Tabela 20 – Consumo de açúcares e geração de etanol usando 0,01 g de células de *Saccharomyces cerevisiae* CAT-1.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	3,295 ± 0,166	3,651 ± 0,182	ND
24	2,141 ± 0,178	2,316 ± 0,184	ND
48	1,971 ± 0,134	2,101 ± 0,020	ND
60	2,018 ± 0,049	2,160 ± 0,056	ND

Tabela 21 – Consumo de açúcares e geração de etanol usando 0,02 g de células de *Spathaspora passalidarum*.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	2,735 ± 0,219	3,035 ± 0,246	ND
24	1,745 ± 0,224	1,971 ± 0,258	ND
48	1,873 ± 0,053	2,125 ± 0,053	ND
60	1,921 ± 0,009	2,189 ± 0,005	ND

Tabela 22 – Consumo de açúcares e geração de etanol usando 0,01 g de células de *Spathaspora passalidarum*.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	2,650 ± 0,164	2,948 ± 0,183	ND
24	1,829 ± 0,114	2,048 ± 0,144	ND
48	1,914 ± 0,031	2,144 ± 0,035	ND
60	1,902 ± 0,011	2,127 ± 0,018	ND

TESTE 2 – Fermentação sem fracionamento, mantendo a biomassa e o caldo no frasco.

Tabela 23 – Consumo de açúcares e geração de etanol usando 0,01 g de células de *Saccharomyces cerevisiae* Innova® Force ADY.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	6,000 ± 0,217	5,907 ± 0,157	ND
24	6,089 ± 0,268	5,930 ± 0,912	ND
48	6,178 ± 0,217	5,996 ± 0,102	0,258 ± 0,066
72	3,177 ± 1,603	4,270 ± 0,880	2,028 ± 0,977

Tabela 24 – Consumo de açúcares e geração de etanol usando 0,01 g de células de *Saccharomyces cerevisiae* CAT-1.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	5,852 ± 0,069	5,560 ± 0,108	ND
24	6,068 ± 0,018	5,721 ± 0,031	ND
48	6,145 ± 0,057	5,765 ± 0,029	ND
72	6,244 ± 0,058	6,035 ± 0,304	ND

Tabela 25 – Consumo de açúcares e geração de etanol usando 0,01 g de células de *Spathaspora passalidarum*.

Tempo (h)	Glicose (g/L)	Xilose (g/L)	Etanol (g/L)
0	5,840 ± 0,183	5,591 ± 0,237	ND
24	5,815 ± 0,085	5,684 ± 0,195	ND
48	5,858 ± 0,011	5,751 ± 0,155	ND
72	5,939 ± 0,033	5,784 ± 0,146	ND

APÊNDICE C – Teste de sinergismo entre os extratos de *C. cubensis* e *T. pinophilus* em casca de soja.

Tabela 26 – Atividades dos extratos individuais e da mistura Atividades enzimáticas dos extratos individuais de *C. cubensis* e *T. pinophilus* crescidos em casca de soja e da mistura entre eles.

Enzima	*C. cubensis* (U)	*T. pinophilus* (U)	Mix esperado (U)	Mix real (U)
FPase (U/mL)	0,0036 ± 0.0003	0,0036 ± 0.0003	0,0072	0,0090 ± 0,0054
Endoglucanase (U/mL)	0,1470 ± 0,0130	0,1650 ± 0,0030	0,3120	0,2320 ± 0,0410
Xilanase (U/mL)	0,2340 ± 0,0100	0,2270 ± 0,0120	0,4610	0,4330 ± 0,0170
β-Glicosidase (U/mL)	0,0310 ± 0,0010	0,0100 ± 0,0002	0,0410	0,0340 ± 0,0005
β-Xilosidase (U/mL)	0,0018 ± 0,004	0,0038 ± 0,0002	0,0056	0,0054 ± 0,0001