CLARA RESENDE DE SOUZA CASTRO

BIOTECHNOLOGICAL APPLICATIONS OF A CRUDE ENZYMATIC EXTRACT FROM *Penicillium brevicompactum*

LAVRAS - MG
2025

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Tese submetida à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para obtenção do título de Doutor.

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2025

Ficha Catalográfica elaborada pelo Sistema de Geraçãode Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Resende de Souza Castro, Clara.
Biotechnological applications of a crude enzymatic extract from *Penicillium brevicompactum* / Clara Resende de Souza Castro. – Lavras : UFLA, 2025.
110 p. : il.

Tese (Doutorado) - Universidade Federal de Lavras, 2025.
Orientadora: Cristina Ferreira Silva
Coorientador: Filippe Elias De Freitas Soares
Bibliografia.

1. Biorrefinaria. 2. Enzimas. 3. Poluentes orgânicos emergentes. 4. Controle biológico. I. Ferreira Silva, Cristina. II. De Freitas Soares, Filippe Elias. III. Universidade Federal de Lavras. IV. Título.

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BIOTECHNOLOGICAL APPLICATIONS OF A CRUDE ENZYMATIC EXTRACT FROM *Penicillium brevicompactum*

APLICAÇÕES BIOTECNOLÓGICAS DO EXTRATO BRUTO ENZIMÁTICO DE *Penicillium brevicompactum*

Tese submetida à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para obtenção do título de Doutor.

APROVADA em 30 de maio de 2025.

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LAVRAS - MG
2025

Aos meus pais.
Dedico.

AGRADECIMENTOS

Agradeço a Deus pela capacidade de ser resiliente frente às adversidades;

A minha família, em especial mãe, pai e irmão, pelo apoio incondicional;

A minha cachorra, Gaia, pela companhia eterna, principalmente durante as noites viradas;

A minha orientadora, Cristina, pelo incentivo diário, confiança em meu trabalho, parceria e apoio em quase 10 anos de laboratório;

Ao meu coorientador, Filippe, pelos ensinamentos, paciência, disponibilidade, confiança em meu trabalho e, principalmente, por ter aberto as portas do seu laboratório para mim;

Aos amigos do laboratório, em especial Danilo, Tai, Anna, Cris, Nayara, Willian e Victor;

Aos membros do NEMAI;

Aos amigos de anos, em especial Bertô, Iana, Sérgio, Léo, Isa, Rafinha e Ian;

Ao Adrian, pela companhia diária e por toda a ajuda no laboratório;

A todos os técnicos, servidores e docentes da Universidade Federal de Lavras, em especial à Cidinha;

Aos amigos e orientadores da Holanda, por todo o auxílio e companhia durante a experiência do doutorado sanduíche;

Aos membros do Labba, por toda a ajuda durante a realização deste trabalho;

Por fim, a todos que contribuíram direta e indiretamente com a realização deste trabalho;

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Ao Programa de Pós-graduação em Microbiologia Agrícola.

“Slow down, you're doing fine

You can't be everything you wanna be before your time”

(Billy Joel - Vienna)

RESUMO

Poluentes ambientais como atrazina e desetilatrazina, um herbicida comumente utilizado e seu produto de transformação, juntamente com o acúmulo de resíduos lignocelulósicos da agricultura, representam desafios que ameaçam a saúde, a biodiversidade e a produção sustentável de alimentos. Este estudo analisa como o fungo *Penicillium brevicompactum*, quando cultivado em bagaço de cana-de-açúcar, pode servir como uma maneira prática e econômica de combater a poluição e produzir enzimas úteis. O processo de fermentação em estado sólido foi otimizado, mantendo o teor de umidade em 65% por sete dias, o que resultou em altos rendimentos de enzimas importantes, incluindo ligninases e uma variedade de enzimas degradadoras de parede c como celular, como carboximetilcelulase, celulase total, xilanase, pectinase, avicelase, β-glicosidase, protease e quitinase. O extrato bruto enzimático apresentou grande potencial para diversas aplicações. Notavelmente, tanto o extrato bruto quanto o dialisado foram capazes de degradar cerca de 75% da atrazina e da desetilatrazina em apenas 21 dias, oferecendo um método alternativo para a remoção de poluentes. A análise do bagaço de cana-de-açúcar após a fermentação demonstrou alterações estruturais e químicas significativas, indicando conversão efetiva de biomassa. Os extratos também demonstraram atividade antifúngica, inibindo mais de 60% de diferentes espécies de fungos fitopatogênicos, e se mostraram eficazes contra nematoides, reduzindo as larvas de *Panagrellus* sp. em até 75%. Esses resultados destacam o potencial do uso de *P. brevicompactum* juntamente com resíduos agrícolas para a produção sustentável de enzimas e extratos benéficos. Esse método pode levar a soluções efetivas para a biorremediação de ambientes, bem como promover uma agricultura mais sustentável, transformando resíduos em produtos de valor agregado e abrangendo a criação de produtos ecologicamente corretos para a proteção das lavouras. No futuro, mais pesquisas devem se concentrar em ampliar a escala desses processos e testar sua eficácia em ambientes agrícolas reais para facilitar o uso mais amplo na gestão ambiental e nas práticas agrícolas.

Palavras-chave: agricultura sustentável; poluentes orgânicos emergentes; biorremediação; economia circular.

ABSTRACT

Environmental pollutants, such as atrazine and desethyl-atrazine, a herbicide commonly used in agriculture and its transformation product, along with the accumulation of lignocellulosic residues from agriculture, pose serious challenges that threaten human health, biodiversity, and sustainable food production. This study examines how the fungus *Penicillium brevicompactum*, when cultivated on sugarcane bagasse, can provide a practical and cost-effective solution to combat pollution while producing useful enzymes. The solid-state fermentation process was optimized by maintaining the moisture content at 65% for seven days, resulting in high yields of critical enzymes, including ligninases and various cell wall-degrading enzymes, such as carboxymethyl cellulose, total cellulase, xylanase, pectinase, avicelase, β-glucosidase, protease, and chitinase. The crude enzyme extract demonstrated significant potential for a range of applications. Notably, both the crude extract and the dialysate were able to degrade approximately 75% of atrazine and desethyl-atrazine within just 21 days, offering an alternative method for pollutant removal. Analysis of the sugarcane bagasse after fermentation revealed significant structural and chemical changes, indicating effective biomass conversion. The extracts also exhibited antifungal properties, inhibiting over 60% of various species of phytopathogenic fungi, and proved effective against nematodes, reducing the larvae of *Panagrellus* sp. by up to 75%. These results underscore the potential of using *P. brevicompactum* in conjunction with agricultural residues to sustainably produce beneficial enzymes and extracts. This approach could lead to effective solutions for environmental bioremediation and promote more sustainable agricultural practices, transforming waste into value-added products while creating environmentally friendly options for crop protection. Future research should focus on scaling up these processes and testing their effectiveness in real agricultural settings to facilitate broader implementation in environmental management and agricultural practices.

Keywords: sustainable agriculture; emerging organic pollutants; bioremediation; circular economy.

INDICADORES DE IMPACTO

A pesquisa explora o uso do bagaço de cana-de-açúcar como substrato para a produção de enzimas degradadoras da parede celular utilizando *Penicillium brevicompactum*. Essa abordagem não visa apenas reduzir custos, mas também ampliar a disponibilidade de biocatalisadores de alto valor agregado. Em nível social e ambiental, essa iniciativa pode abordar a questão da má gestão de resíduos agrícolas. Ao promover o uso de subprodutos do agronegócio, pode contribuir para a melhoria da qualidade do solo e da água por meio da degradação de poluentes orgânicos, impactando positivamente a saúde pública e o acesso à água potável. Além disso, a utilização de um extrato enzimático bruto para o controle de pragas agrícolas contribui para uma prática agrícola mais sustentável e viável. Do ponto de vista tecnológico e econômico, o desenvolvimento de processos de fermentação mais eficientes e acessíveis pode impulsionar a inovação em diversos setores — da produção de biocombustíveis à bioconversão de resíduos. Isso está alinhado aos princípios de uma economia circular. O projeto também possui um caráter extensionista, pois possui o potencial de transferir tecnologia para produtores rurais, agroindústrias e empresas de biotecnologia, especialmente em regiões onde a cana-de-açúcar é cultivada, beneficiando, por fim, agricultores, técnicos e comunidades locais. A área de foco desta pesquisa abrange áreas rurais do Brasil conhecidas pela produção de açúcar e etanol, beneficiando diretamente grupos como trabalhadores rurais, técnicos agrícolas e pesquisadores. Também envolve professores universitários e estudantes. Os impactos são categorizados principalmente nos temas Meio Ambiente, Tecnologia e Produção, Saúde e Trabalho, em consonância com a Política Nacional de Extensão Universitária. Por fim, este projeto está alinhado com diversos Objetivos de Desenvolvimento Sustentável da ONU, em particular o ODS 2 (Fome Zero e Agricultura Sustentável), o ODS 6 (Água Potável e Saneamento), o ODS 12 (Consumo e Produção Responsáveis) e o ODS 13 (Ação Climática). Ao promover soluções de baixo custo para gestão de resíduos e contribuir para a proteção ambiental e o desenvolvimento socioeconômico da região, o projeto demonstra um potencial que pode ser replicado em outros contextos agrícolas, tanto em nível nacional quanto internacional.

IMPACT INDICATORS

The research investigates the use of sugarcane bagasse as a substrate for producing cell wall-degrading enzymes using *Penicillium brevicompactum*. This approach aims not only to reduce costs but also to increase the availability of high-value biocatalysts. On a social and environmental level, this initiative addresses the challenge of poor agricultural waste management. By promoting the use of agribusiness by-products, it can enhance soil and water quality through the degradation of organic pollutants, which positively impacts public health and accessibility to drinking water. Additionally, utilizing a crude enzyme extract for agricultural pest control contributes to more sustainable and viable agricultural practices. From a technological and economic standpoint, developing more efficient and accessible fermentation processes can drive innovation across various sectors—ranging from biofuel production to waste bioconversion. This aligns with the principles of a circular economy. The project also has an extensionist component, with the potential to transfer technology to rural producers, agribusinesses, and biotechnology companies, especially in regions where sugarcane is cultivated, ultimately benefiting farmers, technicians, and local communities. The focus of this research encompasses rural areas in Brazil recognized for sugar and ethanol production, directly benefiting groups such as rural workers, agricultural technicians, and researchers, as well as university professors and students. The impacts are categorized primarily under the themes of Environment, Technology and Production, Health and Labor, in line with the National Policy for University Extension. Finally, this project aligns with several UN Sustainable Development Goals, particularly SDG 2 (Zero Hunger and Sustainable Agriculture), SDG 6 (Clean Water and Sanitation), SDG 12 (Responsible Consumption and Production), and SDG 13 (Climate Action). By promoting low-cost waste management solutions and contributing to environmental protection and socioeconomic development in the region, the project demonstrates potential for replication in other agricultural contexts, both nationally and internationally.

LISTA DE SIGLAS

CMCase	Carboximetilcelulase
CONAMA	Conselho Nacional Do Meio Ambiente
CWDE	Cell-Wall Degrading Enzymes
FES	Fermentação Em Estado Sólido
FPase	Filter Paper Cellulase
FTIR	Fourier-Transform Infrared Spectroscopy
GDP	Gross Domestic Product
HPLC	High Performance Liquid Chromatography
LiP	Lignina Peroxidase
MnPase	Manganês Peroxidase
ONU	Organização das Nações Unidas
PIB	Produto Interno Bruto
SCB	Sugarcane Bagasse
SEM	Scanning Electron Microscopy

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PRIMEIRA PARTE

1 INTRODUÇÃO

Anualmente, grandes quantidades de resíduos lignocelulósicos são geradas, especialmente em países nos quais o agronegócio exerce um papel relevante na economia. O manejo inadequado desses resíduos, como o bagaço de cana-de-açúcar, pode causar impactos ambientais negativos devido a práticas de descarte inadequadas. A cana-de-açúcar é uma das culturas agrícolas mais amplamente cultivadas no mundo, com produção voltada principalmente para alimentos, bebidas e bioetanol, resultando em grandes volumes de subprodutos lignocelulósicos. Entre esses subprodutos, o bagaço se destaca por seu potencial para aplicações de valor agregado, especialmente nos setores de bioenergia e biotecnologia, em razão de sua composição química favorável.

Uma alternativa promissora consiste na utilização do bagaço como substrato em processos de fermentação em estado sólido (FES), nos quais microrganismos decompõem a biomassa para a obtenção de produtos de interesse, como enzimas e metabólitos. A FES apresenta vantagens econômicas e ambientais, além de possibilitar a produção de enzimas que atuam na degradação da parede celular vegetal, como celulases, hemicelulases, ligninases e pectinases. Essas enzimas possuem diversas aplicações industriais, incluindo a melhoria da compostagem, a potencialização de biofertilizantes, o aumento da digestibilidade de silagens e o processamento de extratos vegetais.

Além das enzimas, a FES pode originar outros produtos biológicos, como surfactantes, biopesticidas e antibióticos. Contudo, a viabilidade comercial da FES ainda enfrenta desafios relacionados aos custos de produção e à necessidade de otimização do crescimento microbiano e da eficiência enzimática, fatores que dependem de variáveis como temperatura, umidade, tempo de incubação, aeração e oferta de nutrientes.

Os extratos enzimáticos obtidos a partir do bagaço também apresentam potencial para aplicações em biorremediação e no controle biológico de pragas. Enzimas ligninolíticas, como peroxidases e lacases, podem contribuir para a degradação de poluentes orgânicos persistentes, incluindo resíduos de herbicidas que oferecem riscos à saúde e ao meio ambiente. A biorremediação enzimática representa uma alternativa eficiente e direcionada para a desintoxicação de contaminantes em diferentes contextos ambientais. Paralelamente, enzimas como quitinases e proteases, produzidas por fungos, desempenham um papel importante no controle biológico de pragas agrícolas, oferecendo alternativas sustentáveis aos pesticidas convencionais. Esses extratos podem inibir o crescimento de fungos fitopatogênicos e nematoides, colaborando com práticas de manejo integrado de pragas.

Apesar do avanço nesse campo, são necessárias pesquisas adicionais para definir as condições ideais de fermentação, aprimorar a estabilidade e a atividade das enzimas e explorar novas cepas fúngicas com potencial biotecnológico. Neste contexto, o presente estudo busca: (i) otimizar as condições para a produção de enzimas-chave, como celulases, xilanase, pectinase, lignina peroxidase, lacase, manganês peroxidase, quitinase e protease, por *Penicillium brevicompactum* utilizando bagaço de cana como substrato; (ii) caracterizar quimicamente e morfologicamente o bagaço durante a fermentação; (iii) avaliar a cinética de degradação de poluentes como a atrazina e a desetilatrazina; e (iv) investigar a atividade biológica de extratos brutos frente a fungos fitopatogênicos e nematoides.

A proposta contribui para a valorização de resíduos agroindustriais, a promoção de práticas sustentáveis de manejo e a ampliação de soluções para a recuperação ambiental e a produtividade agrícola.

2 REFERENCIAL TEÓRICO

2.1. Agroindústria, resíduos lignocelulósicos e o bagaço de cana

A agroindústria brasileira é responsável por uma parcela considerável do Produto Interno Bruto (PIB) brasileiro, aproximadamente 23,2% (CEPEA, 2024) com destaque para produção de café, soja, cana-de-açúcar, mandioca, entre outros. Complementando, a Organização das Nações Unidas para a Alimentação e a Agricultura apontou que o Brasil poderá ser responsável por metade da produção mundial alimentícia até 2050, na qual deverá ser ampliada em até 60% (FAO, 2017). Em 2024, por exemplo, o país atingiu uma produção de cana-de-açúcar equivalente a 676,96 milhões de toneladas, tornando-se recorde na produção de açúcar. Grande parte é destinada para produção de etanol (CONAB, 2025).

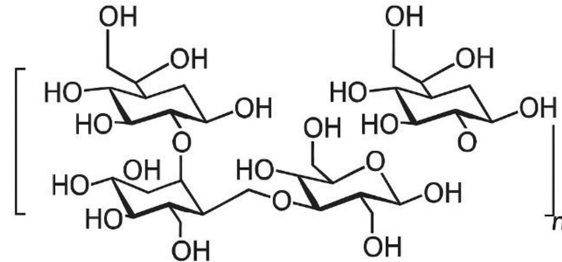
Ainda que possua tamanha relevância, a produção de cana-de-açúcar, no Brasil, é responsável por gerar cerca de 26% toneladas de bagaço para cada tonelada de cana produzida, sendo considerada predominante entre os demais resíduos produzidos pela agroindústria (NOVA CANA, 2014). É inegável a importância do setor agroindustrial para economia brasileira uma vez que gera renda e mantém o homem no campo. No entanto, embora a importância seja positiva em relação à produção de alimentos e ao impacto na economia brasileira, há o agravamento dos problemas ambientais, tais como o aumento do desmatamento, poluição, mudanças climáticas e a geração de toneladas de resíduos todos os anos (Viola & Mendes, 2022).

Diante disso, o maior desafio é agregar valor aos resíduos, de forma que eles possam ser reutilizados ou aplicados em outros processos. Atualmente, muitos destes resíduos são estudados para aplicação em processos de biorrefinaria, biorremediação de áreas contaminadas, produção de biogás, produção de painéis de construção, produção de biocombustível, como substrato para produção de cogumelos e como ração bovina (Nizami et al., 2017; Yaashikaa & Kumar, 2022). Entretanto, mesmo com estes usos, há ainda muito resíduo que não é utilizado ou que o processo de reutilização ainda é custoso.

Os resíduos agroindustriais são, em sua maioria, compostos de celulose, hemicelulose e lignina (Figuras 1, 2 e 3), além de cinzas, carbono, nitrogênio etc. Os três primeiros polímeros são encontrados em maiores proporções nesses materiais – embora a quantidade possa variar de espécie para espécie - e, por isso, estes resíduos podem ser chamados de “materiais lignocelulósicos”. A celulose e a hemicelulose são polímeros formados por diferentes açúcares,

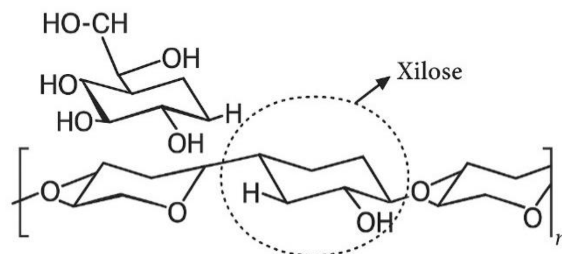
como glicose e xilose, e a lignina é um polímero aromático sintetizado por precursores de fenilpropanoides (Hiloidhari et al., 2020; Segers et al., 2024).

Figura 1 - Representação estrutural da celulose



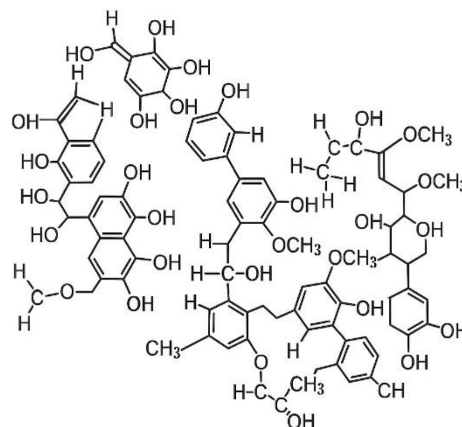
Fonte: Elaborado pelo autor (2025)

Figura 2 - Representação estrutural da hemicelulose



Fonte: Elaborado pelo autor (2025)

Figura 3 - Representação estrutural da lignina



Fonte: Elaborado pelo autor (2025)

Quando se conhece a composição química destes materiais, torna-se mais fácil prever as possíveis aplicações biotecnológicas. A biomassa lignocelulósica é considerada muito estável devido à complexidade das moléculas ali presentes, especialmente a presença de anéis aromáticos, que dificulta a hidrólise e a oxidação dos polímeros e a utilização de técnicas de conversão destes materiais para liberação dos açúcares e fenóis presentes nas moléculas de lignina, celulose e hemicelulose torna-se fundamental (Goodman, 2020).Uma maneira de

realizar a conversão é através da fermentação, ocorrendo uma transformação bioquímica no resíduo. Este processo é capaz de adicionar um valor agregado à biomassa lignocelulósica através de hidrólises enzimáticas ou de sacarificação, que são capazes de modificar as frações poliméricas (lignina, celulose e hemicelulose), tornando-as menos complexas. Os microrganismos conseguem converter o produto da fermentação em álcool, ácidos orgânicos, alcenos, lipídios ou outras moléculas de interesse (Geng et al., 2023).

Um exemplo de bioconversão da biomassa lignocelulósica muito utilizado é a do bagaço de cana. O bagaço de cana é um dos principais subprodutos derivado da cana-de-açúcar e destaca-se pelas suas diversas aplicabilidades, como a produção de glicose, biocombustíveis e biofertilizantes (Ruan et al., 2024). Para tal, as moléculas são hidrolisadas por ação de químicos ou de enzimas. A hidrólise enzimática pode ser considerada uma opção mais sustentável, uma vez que é realizada por microrganismos e, este fato, muitas das vezes, torna o processo menos oneroso (Ramos et al., 2021; Yaverino-Gutierrez et al., 2024).

A necessidade emergente de se converter ou degradar os resíduos agroindustriais se dá pela quantidade em que são produzidos. O bagaço tem um alto valor em relação aos outros resíduos, uma vez que pode ser utilizado para produção de biocombustíveis, energia elétrica, furfural (F. Santos et al., 2020),como substrato para síntese de enzimas microbianas, como celulases, xilanases e lacases (Ferreira et al., 2018; Ogunyewo et al., 2020), como material alternativo em construções civis (Dawoud et al., 2020). Além de ser utilizado como substrato, o bagaço de cana também é utilizado como suporte de imobilização de células para produção de compostos, como o xilitol (Antunes et al., 2021).

Ainda que gerado em larga escala, este subproduto pode ser considerado versátil devido as inúmeras possibilidades de agregação de valor em produtos e reaproveitamento (F. Santos et al., 2020). Portanto, faz-se necessário o aperfeiçoamento de metodologias que envolvam o bagaço de cana e microrganismos, principalmente para síntese de enzimas, a fim de diminuir os custos de processamento e viabilizar o processo em escala industrial.

2.2. Poluentes orgânicos

É notável a importância da agricultura e dos sistemas de produção alimentares em todo o mundo. Por isso, instituições de renome como a Organização das Nações Unidas para Alimentação e Agricultura buscam alternativas para mitigar o impacto gerado por essas atividades e assegurar a segurança alimentar das atuais e futuras gerações. Um dos desafios decorrentes da intensa atividade agropecuária é o uso inadequado de insumos agrícolas,

incluindo defensivos agrícolas e antibióticos, não apenas nos sistemas de produção pecuários voltados para o uso animal. O uso inadequado desses compostos pode resultar na acumulação de resíduos tóxicos e persistentes no ambiente, afetando diretamente os ecossistemas terrestres e aquáticos, além das atividades produtivas (FAO, 2023).

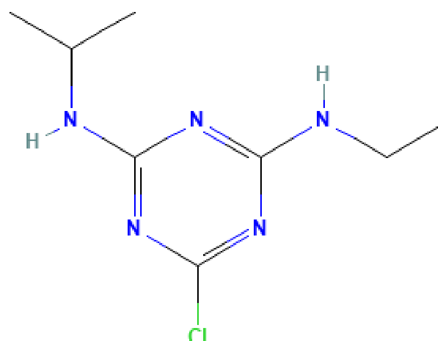
2.2.1. Defensivo agrícola: atrazina

Os defensivos agrícolas químicos são utilizados na agricultura para o controle de doenças e para prevenção de uma série de agentes como bactérias, vírus, fungos, insetos, nematoides e ervas daninhas. O uso destes químicos foi responsável pelo aumento da produção e pela diminuição das perdas nas colheitas. Além disso, foi capaz de aumentar a disponibilidade de alimentos e, consequentemente, suprir a demanda necessária. Estima-se que aproximadamente três bilhões de quilos de defensivos químicos são utilizados todos os anos, sendo notável a importância de seu uso (Sharma et al., 2020).

Contudo, o uso inadequado destes pode trazer consequências severas para o meio ambiente e para a saúde humana. Seu potencial bioacumulativo altera os ambientes bióticos e abióticos, afetando o equilíbrio do ecossistema. Ademais, quando utilizados em doses excessivas, podem alterar o funcionamento das células eucaróticas e procarióticas (Qadir et al., 2017; Rani et al., 2020; Sharma et al., 2020), como é o caso da atrazina.

A atrazina (2-cloro-4-etilamino-6-isopropilamino-1,3,5-triazina) é um herbicida amplamente utilizado no mundo todo, que age no combate de ervas daninhas e gramíneas de culturas de cana-de-açúcar, trigo e milho (NPIC, 2020). Pertencente a classe das triazinas, o químico tem uma estrutura que compreende um anel aromático com átomos de carbono e nitrogênio (Figura 4). Dentre as propriedades químicas da substância, destaca-se a baixa solubilidade em água e a capacidade de acumulação em altas concentrações (42,8 µg/L), principalmente nos ecossistemas aquáticos e no solo (de Albuquerque et al., 2020).

Figura 4 - Representação química da estrutura da atrazina



Fonte: PubChem (Acesso em: maio de 2025)

Sucintamente, o herbicida atua na inibição da fotossíntese ao bloquear o transporte de elétrons no fotossistema II, reduzindo a produção de ATP e NADPH, bem como a produtividade do processo de fixação de CO_2. Devido ao uso em larga escala, é comum encontrar resíduos do pesticida nos solos e cursos hídricos e, embora certa quantidade seja permitida, faz-se necessário o manejo correto deste. A resolução CONAMA nº 396 de 2008 determina a concentração máxima de atrazina permitida em águas doces de até 2 µg/L, além disso, estudos demonstram que o pesticida se mantém no solo após a aplicação, e possui uma meia vida variável e dependente do manejo (BRASIL, 2008; NPIC, 2020).

Utilizada em larga escala, a atrazina apresenta uma capacidade de persistência na natureza devido a sua meia vida - que pode variar de 21 dias a um ano -, o que ocasiona uma acumulação em níveis considerados acima do permitido (2 µg/L) (Jakinala et al., 2019). Apesar de existirem diversos tratamentos físico-químicos para remoção da atrazina, os tratamentos biológicos vêm ganhando espaço devido a possibilidade de se utilizar microrganismos e seus metabólitos na degradação deste composto (Yang et al., 2018). Diante disso, é importante considerar alternativas que envolvam a manutenção correta de químicos no meio ambiente e meios de biorremediar as áreas afetadas.

2.3. Enzimas degradadoras de parede celular

De acordo com Copeland (2023): "as enzimas são catalizadores biológicos, de natureza principalmente proteica que participam de várias reações bioquímicas, tendo como papel fundamental o controle metabólico". Baseado na natureza das reações químicas, as enzimas podem ser divididas em seis classes: ligases, isomerases, liases, hidrolases, transferases e

oxidorredutases (IUPAC-IUB Commission on Biochemical Nomenclature Tentative Rules, 1967).

A comissão de enzimas (EC) codifica cada enzima de acordo com sua classe, seu modo de ação, o seu aceptor e, por fim, o seu conjunto de características reacionais (*BRENDA Enzyme Database*, 2025). Desta forma, a classificação das principais enzimas degradadoras de parede são:

Lacase: EC 1.10.3.2

1 (Oxidorredutase). 10 (Atua na catálise da oxidação de moléculas difenóis e substâncias afins como doadores). 3 (Aceptor: oxigênio). 2 (São um grupo de enzimas multi-cobre com baixa especificidade que atuam em o- e p- quinois e atuam também em aminofenois e fenilenediamina).

Manganês peroxidase: EC 1.11.1.3

1 (Oxidorredutase). 11 (Atua na catálise da oxidação de moléculas orgânicas fenólicas e afins na presença de manganês). 1 (Aceptor: peróxido de hidrogênio). 3 (São um grupo de enzimas heme que atuam estritamente em substratos fenólicos).

Lignina peroxidase: EC 1.11.1.14

1 (Oxidorredutase). 11 (Atua na catálise da oxidação de moléculas orgânicas fenólicas e aromáticas não fenólicas, na qual o H_2O_2 é o principal doador de elétrons). 1 (Aceptor: peróxido de hidrogênio). 14 (São um grupo de enzimas heme que atuam em substratos fenólicos e aromáticos não fenólicos).

Carboximetilcelulases: EC 3.2.1.203

3 (Hidrolase). 2 (Atua na catálise da hidrólise das ligações glicosídicas presentes na celulose). 1 (Aceptor: água). 203 (São um grupo de enzimas que atuam na catálise da hidrólise do interior das cadeias de celulose, transformando as ligações glicosídicas em unidades de glicose).

Xilanase: EC 3.2.1.8

3 (Hidrolase). 2 (Atua na catálise da hidrólise das ligações glicosídicas presentes na hemicelulose). 1 (Aceptor: água). 8 (São um grupo de enzimas que atuam na catálise da hidrólise do interior das cadeiras de hemicelulose, transformando as ligações glicosídicas dos polissacarídeos de xilana, em unidades de xilose).

β-glicosidase: EC 3.2.1.21

3 (Hidrolase). 2 (Atua na catálise da hidrólise das ligações glicosídicas β em β-D-glicosídeos, celobiose e oligossacarídeos). 1 (Aceptor: água). 21 (São um grupo de enzimas que catalisam a hidrólise de β-glicosídeos, liberando glicose como produto final).

Pectinase: EC 3.2.1.15

3 (Hidrolase). 2 (Atua na catálise da hidrólise das ligações glicosídicas presentes na pectina, um polissacarídeo da parede celular vegetal). 1 (Aceptor: água). 15 (São um complexo enzimático composto por pectinesterase, poligalacturonase e pectinliase, que catalisam a hidrólise pectina em ácidos galacturônicos e oligossacarídeos).

Avicelase: EC 3.2.1.91

3 (Hidrolase). 2 (Atua na catálise da hidrólise das ligações glicosídicas presentes na celulose microcristalina, como o Avicel). 1 (Aceptor: água). 91 (São um grupo de enzimas que catalisam a hidrólise do interior das cadeias de celulose microcristalina, liberando glicose como produto final).

Quitinase: EC 3.2.1.14

3 (Hidrolase). 2 (Atua na catálise da hidrólise das ligações β-1,4-glicosídicas presentes na quitina, principal componente da parede celular de fungos e exoesqueleto de artrópodes). 1 (Aceptor: água). 14 (São enzimas capazes de catalisar a hidrólise quitina, apresentando atividade ótima em pH ácido e temperatura moderada, com aplicações antifúngicas).

Protease: E.C. 3.4

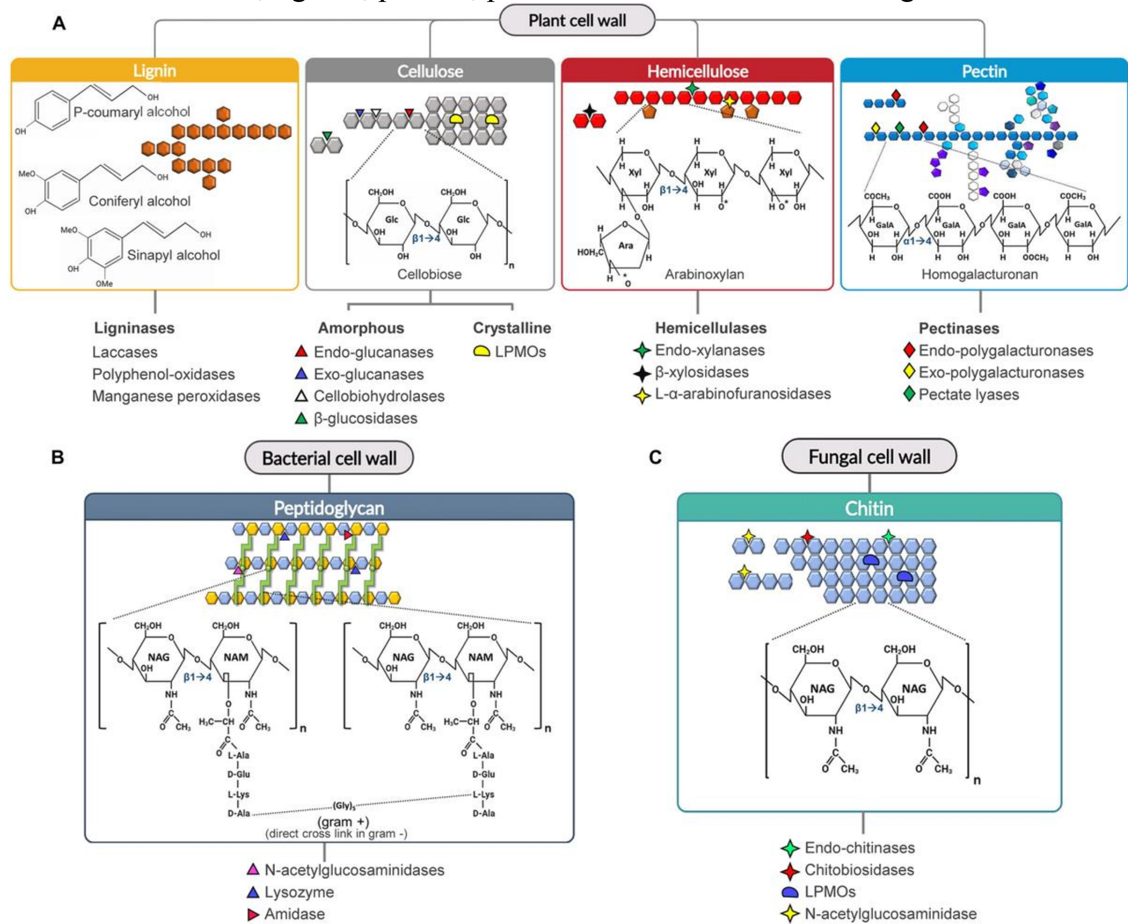
3 (Hidrolase). 4 (Atua na catálise da hidrólise das ligações peptídicas presentes em proteínas). - (Aceptor: água). - (São um grupo de enzimas que catalisam a hidrólise de proteínas em peptídeos menores ou aminoácidos livres, podendo ter especificidade ampla ou restrita, e mecanismos catalíticos variados, como serina, cisteína, aspartato ou metaloproteases).

As enzimas microbianas podem ser sintetizadas por bactérias, fungos filamentosos ou leveduras. Dentre os mais estudados, destacam-se os ascomicetos, os basidiomicetos e as bactérias do gênero *Bacillus* – principalmente sua expressão gênica a fim de elucidar os mecanismos envolvidos na produção enzimática/síntese proteica. Com o avanço das técnicas moleculares e da genômica, uma série de espécies microbianas já são capazes de produzir enzimas de todas as classes, com o foco na produção industrial (El-Gendi et al., 2021; Slimane & El-Hafid, 2024).

Fungos unicelulares como as leveduras são conhecidas pelo seu potencial de uso em diversos setores industriais, por meio da fermentação de bebidas, alimentos, da produção de etanol e elaboração de ração e forragem, além da produção de enzimas e outros metabólitos (El-Gendi et al., 2021). Além das leveduras, os fungos filamentosos também são importantes aliados à biotecnologia, uma vez que atuam na ciclagem de nutrientes através da decomposição, respiração e da produção de biomassa e metabólitos (Meyer et al., 2020). Por isso, podem ser explorados para diversos fins e os metabólitos de maior interesse são ácidos graxos e orgânicos, enzimas, vitaminas, aminoácidos e antibióticos. Dentre os fungos filamentosos, destacam-se os gêneros *Penicillium, Aspergillus* e *Trichoderma* (Boruta et al., 2020; Meyer et al., 2020), e, dentre as leveduras, destacam-se os gêneros *Kluyveromyces* e *Saccharomyces* (Runguphan & Kocharin, 2022). A espécie *Penicillium brevicompactum*, por exemplo, embora pouco relatado na literatura, é considerada versátil e sua fisiologia permite que suas condições de cultivo sejam bem exploradas. No geral, a espécie cresce entre -2 a 30 °C e possui a temperatura ótima de crescimento igual a 23 °C numa faixa de pH entre 3-7 (Pitt, 2006).

A síntese de enzimas degradadoras de parede, como as celulases por *Trichoderma* (Yan et al., 2021), xilanases e lacases por *Penicillium* (de Souza Castro et al., 2024a; Nunes et al., 2024a) para o processo de hidrólise da biomassa lignocelulósica tem se mostrado uma ferramenta indispensável para garantir um processo mais economicamente viável e menos danoso ao meio ambiente (Hu et al., 2024; Nizami et al., 2017). Estas enzimas irão atuar na despolimerização da celulase, hemicelulose e lignina, sendo que existe um tipo para cada um destes polímeros e o alvo de cada uma delas, conforme ilustrado pela Figura 5.

Figura 5 - Enzimas degradadoras de parede e seus alvos em relação aos polímeros celulose, hemicelulose, lignina, pectina, parede celular de bactérias e fungos

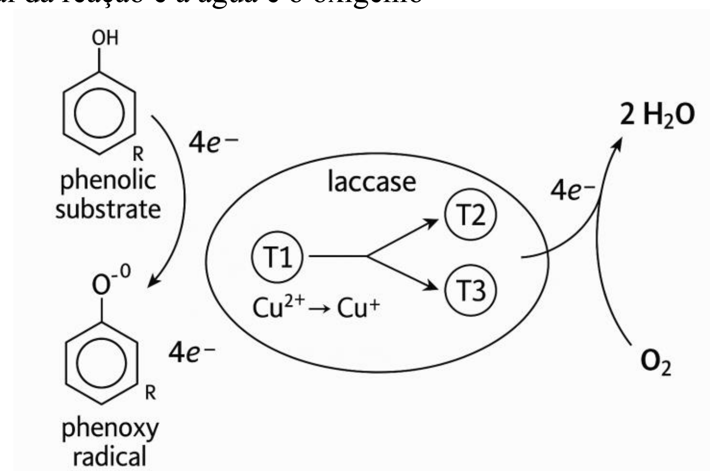


Fonte: Mousavi et al. (2021)

2.3.1. Lacase: mecanismos de ação

As lacases são enzimas multi-cobre oxidases que acoplam a redução do oxigênio em duas moléculas de água com a oxidação de vários substratos fenólicos, arilaminas, anilinas, tióis e lignina. Esta redução é mediada pelos quatro íons de cobre presentes no sítio catalítico enzimático e sua atividade é relacionada à oxidação de compostos fenólicos (S. H. Khatami et al., 2022; Thurston, 1994). A Figura 6 ilustra o mecanismo catalítico da lacase.

Figura 6 - Mecanismo catalítico da lacase sobre uma molécula de substrato fenólico, no qual o produto final da reação é a água e o oxigênio



Fonte: Elaborado pelo autor (2025)

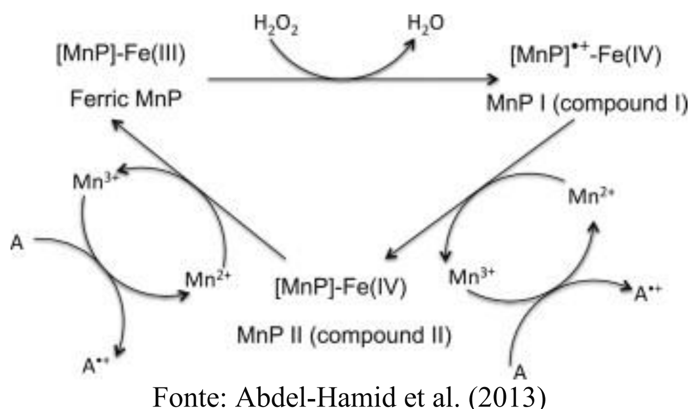
Além da produção por microrganismos como a bactéria *Azospirillum lipoferum* e alguns fungos filamentosos (*Trametes* sp., *Lentinus* sp. e *Pleurotus* sp.), por exemplo, a enzima também pode ser encontrada em algumas espécies de insetos e plantas. Estas enzimas conseguem trabalhar de maneira eficaz em uma série de substratos, sendo considerada uma ótima escolha para aplicações biotecnológicas, como a descoloração e a detoxificação de corantes têxteis e a biorremediação de solos e resíduos contaminantes (Mahuri et al., 2023).

As lacases se destacam pela ampla especificidade, que facilita a ligação da enzima com o composto contaminante alvo. Geralmente, a maneira mais eficiente de utilizá-la é inoculando diretamente o microrganismo produtor no ambiente de interesse, ou pela adição do extrato bruto enzimático (S. H. Khatami et al., 2022).

2.3.2. Manganês peroxidase: mecanismos de ação

A manganês peroxidase é uma enzima responsável por catalisar a oxidação de Mn^{2+} para Mn^{3+} na presença de H_2O_2 (Paszczyński et al., 1985). Os íons de Mn^{3+} atuam como um mediador redox capaz de oxidar estritamente estruturas fenólicas. Embora estruturas não fenólicas não possam ser diretamente oxidadas por esta enzima, existem alguns metabólitos produzidos pela célula que, quando presentes no sistema reacional, também atuam como mediadores, proporcionando então a oxidação de substratos não fenólicos (Chowdhary et al., 2019). A Figura 7 ilustra o ciclo catalítico de manganês peroxidase.

Figura 7 - Ciclo catalítico da manganês peroxidase, no qual ocorre a oxidação de Mn^{2+} para Mn^{3+} mediado por uma molécula de H_2O_2 e o produto final da reação é a água



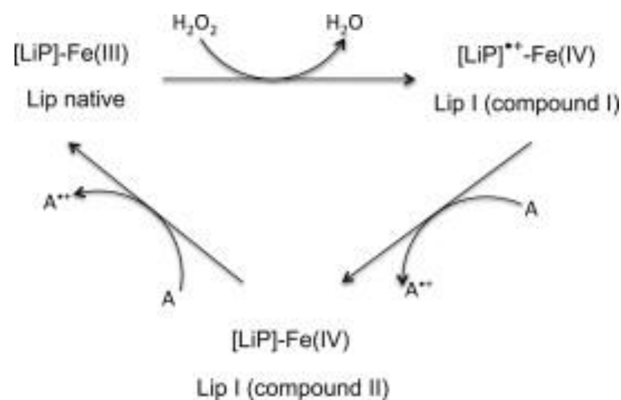
Fonte: Abdel-Hamid et al. (2013)

Estas enzimas são exclusivamente produzidas por microrganismos, especialmente fungos filamentosos do filo Basidiomicota (Kapich et al., 2024; Kheti et al., 2023; Kumar & Chandra, 2018). Elas têm a capacidade de catalisar uma variedade de reações redox em diversos substratos além da lignina, tornando-as catalisadores importantes em várias aplicações industriais, medicinais e biotecnológicas. São frequentemente utilizadas na remediação de solos e águas residuais, além de desempenharem funções de controle biológico, como bioherbicidas (Saikia et al., 2023). Podem ser obtidas por meio de sistemas livres de células ou utilizando o próprio microrganismo produtor da enzima.

2.3.3. Lignina peroxidase: mecanismos de ação

As ligninas peroxidases são enzimas pertencentes ao grupo heme, que a caracterizam com a presença de um átomo de ferro central ligado a quatro átomos de nitrogênio em um anel porfirínico. A presença deste átomo permite a transferência de elétrons e outras reações redox, e isso confere uma ampla gama de funções biológicas, incluindo a catalisação de reações de oxidação, redução e transferência de elétrons (Nguyen et al., 2024; Piontek et al., 2002). A Figura 8 ilustra o ciclo catalítico da lignina peroxidase.

Figura 8 - Ciclo catalítico da lignina peroxidase, mediado por uma molécula de H_2O_2 e o produto final da reação é a água



Fonte: Abdel-Hamid et al. (2013)

São enzimas capazes de catalisar a oxidação de compostos fenólicos e não fenólicos, devido ao maior potencial redox, principalmente quando comparados à lacase e a manganês peroxidase. Nas reações dependentes de lignina peroxidase, não há necessidade de mediadores. Portanto, são capazes de degradar uma diversidade de substratos (Janusz et al., 2013; Nurul-Aliyaa et al., 2023).

Os principais microrganismos produtores desta enzima também pertencem ao grupo dos basidiomicetos, destacando-se as espécies *Arthromyces ramosus, Bjerkandera adusta, Cylindrobasidium evolvens, Ceriporiopsis subvermispora, Daedaleopsis septentrionalis, Phlebia radiata* e *P. chrysosporium* (Kapich et al., 2024; Moiseenko et al., 2023).

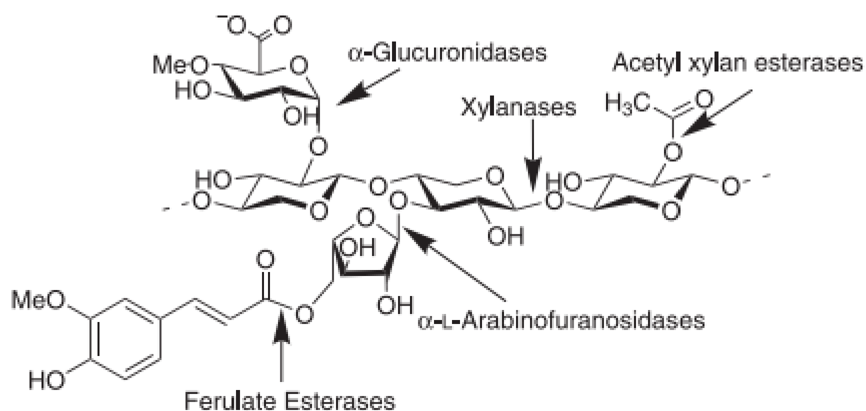
Assim como as outras ligninases, estudos indicam o potencial dessas enzimas na degradação de poluentes ambientais, como corantes têxteis e substâncias disruptoras endócrinas, como o bisfenol A, compostos farmacêuticos, como a tetraciclina, destacando-se seu valor em aplicações para biorremediação de ambientes contaminados com estes compostos (Hussaan et al., 2020; A. K. Singh et al., 2021).

2.3.4. Xilanase: mecanismos de ação

A enzima xilanase (endo-1,4-β-xilanase) desempenha um papel fundamental na hidrólise da xilana, catalisando a hidrólise das ligações β-1,4-xilosídicas entre dois resíduos D-xilopiranosídeos. Essas enzimas compõem o maior grupo de enzimas hidrolíticas envolvidas na degradação da cadeia da xilana, reduzindo seu grau de polimerização e liberando

oligossacarídeos solúveis (Liab et al., 2000; Meagher et al., 1988). A Figura 9 ilustra parte desse processo de degradação da hemicelulose mediado por um complexo enzimático.

Figura 9 - Degradação de xilana mediada pela enzima xilanase e outras enzimas envolvidas no processo



Fonte: Taylor et al. (2006)

As xilanases são majoritariamente produzidas por microrganismos, sendo que as de origem bacteriana se destacam por serem termoestáveis e alcalinas, com um pH ótimo ligeiramente superior ao das enzimas de origem fúngica (Chakdar et al., 2016). Essa característica é relevante em diversas aplicações industriais, especialmente na indústria de papel e celulose, onde são empregadas como agentes de branqueamento, oferecendo uma alternativa ambientalmente correta (Irdawati et al., 2021; Motta et al., 2013).

O uso de xilanases nas indústrias começou em 1980, e cresceu consideravelmente com o passar dos anos. Suas principais aplicações são na preparação de ração animal, na indústria alimentícia, indústria têxtil, em biorrefinarias e na indústria de papel e celulose (Bhardwaj et al., 2019).

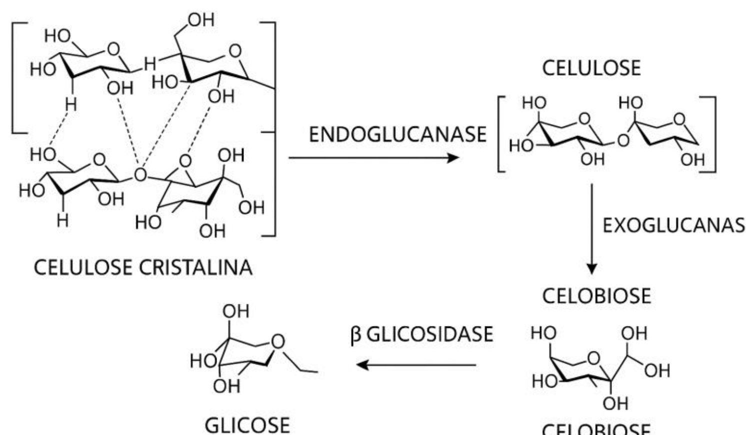
2.3.5. Celulases: mecanismos de ação

As celulases desempenham um papel crucial na catálise da hidrólise das ligações β-1,4 glicosídicas da celulose. Essas enzimas são classificadas em três categorias principais: endoglucanases, exoglucanases e β-glicosidases. Embora todas atuem clivando as ligações de maneira semelhante, a diferença na hidrólise reside na localização do substrato (Knowles et al., 1987; Rigoldi et al., 2018).

A hidrólise da celulose ocorre de forma sinérgica e requer a participação dos três tipos de celulase (Chan et al., 2018). As endoglucanases atuam em regiões de baixa cristalinidade na

fibra de celulose, criando extremidades de cadeia livre. As exoglucanases degradam ainda mais a molécula, removendo as unidades de celobiose das extremidades das cadeias geradas pelas endoglucanases, enquanto as β-glucosidases catalisam a hidrólise da celobiose para produzir glicose livre (Behera & Ray, 2016), conforme ilustrado na Figura 10.

Figura 10 - Mecanismo de ação do complexo enzimático sobre a molécula de celulose



Fonte: Elaborado pelo autor (2025)

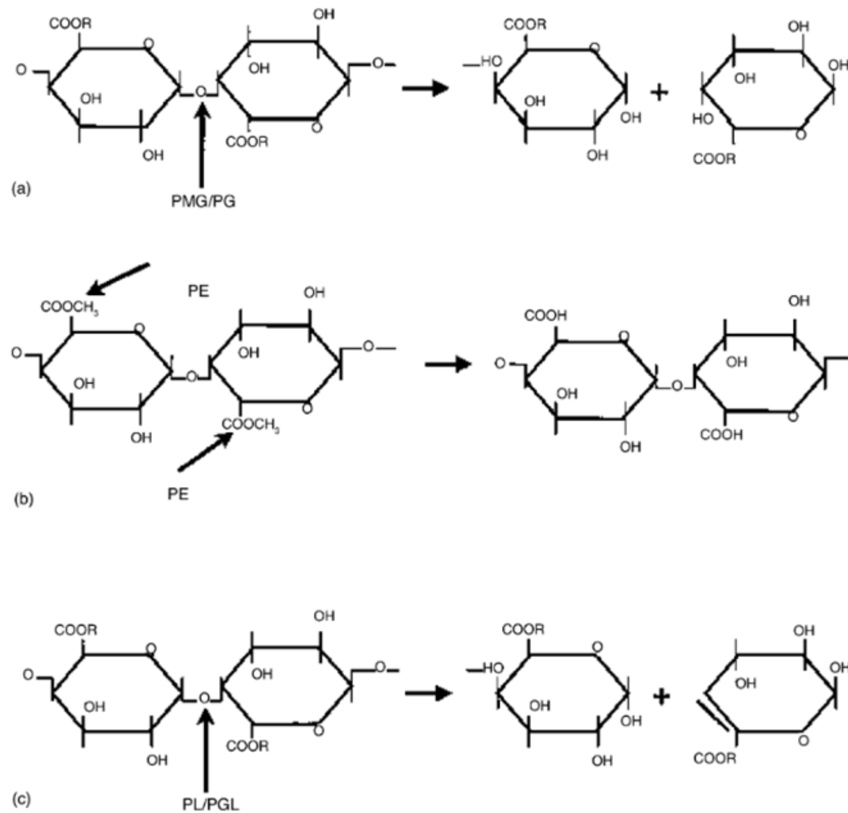
Nos últimos anos, houve um aumento na busca por celulases mais adequadas para aplicação industrial. São enzimas amplamente utilizadas na indústria têxtil, contribuindo para processos como a bioestonagem, que cria uma aparência desbotada em jeans, e a remoção de microfibrilas salientes na superfície de tecidos para reduzir a formação de *pilling* (Santosh U Napte & Prashant P Dixit, 2024). Elas têm uma gama diversificada de aplicações nas indústrias de biocombustíveis, produção de ração animal, fermentação de álcool de grãos, malte e fabricação de cerveja (Sulyman et al., 2020; Verma et al., 2018).

2.3.6. Pectinases: mecanismos de ação

As pectinases são um complexo de enzimas que catalisam a hidrólise de substâncias pécticas. Existem três tipos de pectinases: pectina liases, pectina metil esterases e poligalactorunases (Haile & Ayele, 2022). A Figura 11 mostra o mecanismo de ação de cada uma delas. A pectina liase catalisa a clivagem das ligações α-1,4 entre resíduos de ácido galacturônico esterificados na molécula de pectina. As pectina-metil esterases são responsáveis pela remoção desse grupo metoxila das substâncias pécticas, e esse processo de remoção finalmente produz ácido péctico e metanol. As poligalacturonases atuam no processo de

despolimerização. Assim, elas catalisam a clivagem hidrolítica da cadeia do ácido poligalacturônico na presença de água e a principal função dessa enzima é catalisar a hidrólise da ligação glicosídica alfa 1-4 entre os monômeros galacturônicos (Haile & Ayele, 2022; Shrestha et al., 2021a).

Figura 11 - Mecanismo de ação das poligalactorunases (a); pectina metil esterases (b); e pectina liases (c)



Fonte: Jayani et al. (2005)

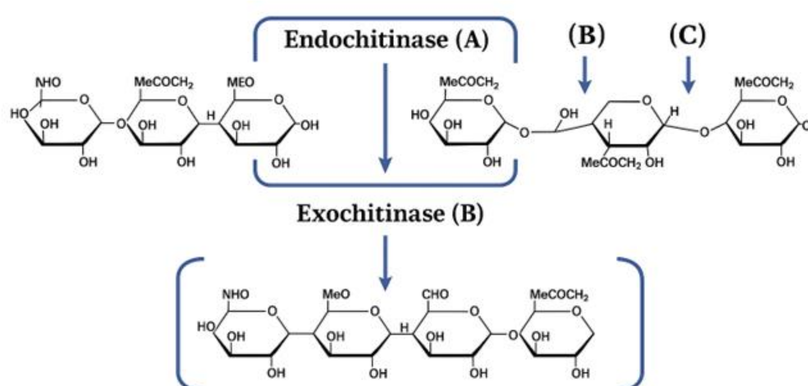
As pectinas possuem várias aplicações nas indústrias. Na alimentícia e cosmética, é utilizada como um agente gelificante, estabilizante e emulsificante (Kaur et al., 2023). Nas indústrias farmacêutica e ambiental, a pectina tem sido usada como hidrogel de pectina em formulações de comprimidos de matriz de liberação controlada e na purificação de água (Shrestha et al., 2021b).

2.3.7. Quitinases: mecanismos de ação

As quitinases são enzimas capazes de catalisar a hidrólise da quitina, um polímero natural resistente e abundante encontrado nas paredes celulares de fungos e nos exoesqueletos

de insetos e crustáceos (Bibra et al., 2017). Estas enzimas podem ser classificadas em dois tipos principais com base em como atuam na quitina: endoquitinases e exoquitinases. As endoquitinases atuam na catálise da hidrólise das cadeias de quitina em pontos internos aleatórios, transformando as moléculas de quitina em fragmentos menores. Em contraste, as exoquitinases catalisam a hidrólise de pequenas unidades de açúcar das extremidades das cadeias de quitina, transformando-as gradualmente em moléculas ainda menores, como diacetilquitobiose ou N-acetilglucosamina (Mahajan et al., 2024). A Figura 12 mostra o mecanismo de ação destas quitinases.

Figura 12 - Mecanismo de ação das endoquitinases (A); exoquitinases (B) sobre a mólecula de quitina



Fonte: Bibra; Krishnaraj; Sani (2017)

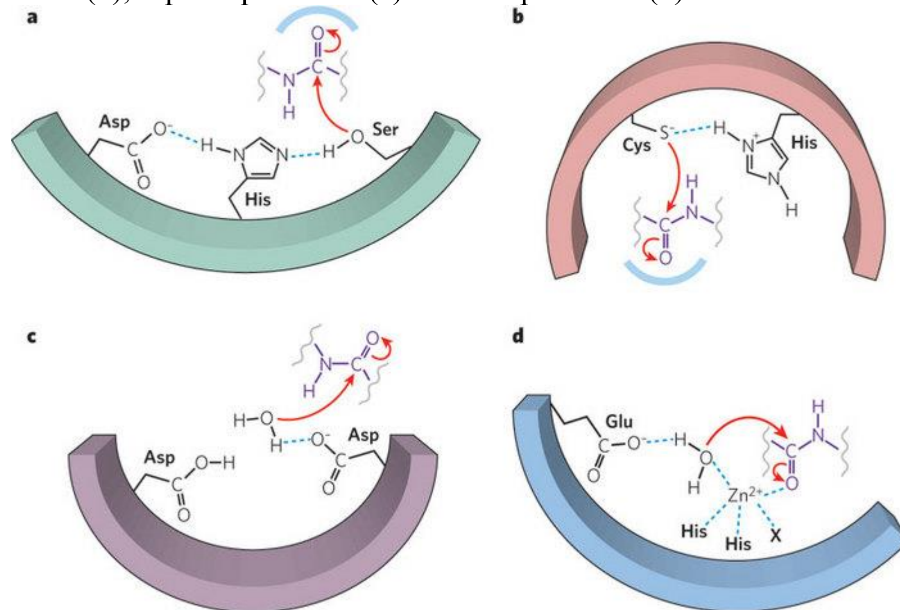
As quitinases possuem ampla aplicabilidade na agricultura, sendo utilizadas como biopesticidas naturais. Na indústria ambiental, são utilizadas para conversão de resíduos ricos em quitina em açúcares (Mahajan et al., 2024). Na indústria média e farmacêutica, a quitina tem sido utilizada como agente antimicrobiano em fármacos (Abu-Tahon & Isaac, 2020).

2.3.8. Proteases: mecanismos de ação

As proteases são enzimas que catalisam a hidrólise de ligações peptídicas em proteínas, por meio de um processo conhecido como proteólise. As proteases são classificadas em quatro tipos principais: serina, cisteína, aspartil e metaloproteases. As duas primeiras utilizam serina ou cisteína, ativadas pela histidina, para formar uma ligação covalente com substratos. Em contrapartida, as duas últimas utilizam aspartato ou íons metálicos, ativando a água como nucleófilo para atacar diretamente as ligações peptídicas (Solanki et al., 2021).

Antigamente, as proteases eram consideradas principalmente enzimas degradadoras de proteínas, porém, esta perspectiva tem evoluído significativamente (Song et al., 2023). A Figura 13 mostra o mecanismo de ação geral das proteases.

Figura 13 - Mecanismo de ação de diferentes proteases: serina proteases solúveis (a), cisteína proteases (b), aspartil proteases (c) e metaloproteases (d)



Fonte: Scitable (2025)

Hoje, as proteases são reconhecidas não apenas como enzimas degradativas, mas também como reguladoras cruciais de vários processos biológicos e alvos importantes para o desenvolvimento de fármacos (Razzaq et al., 2019). Além disso, são enzimas de importância na agricultura, pois atuam no controle de pragas como nematoides e alguns fungos fitopatogênicos, por meio da degradação da parede celular destes (Castro et al., 2023a; Song et al., 2023).

2.4. Extratos fermentados livres de células

Tradicionalmente, os microrganismos são reconhecidos por sua notável capacidade de converter substratos em produtos de alto valor agregado, especialmente por meio de processos fermentativos mediados por esses organismos (Sheldon & Woodley, 2018). No entanto, tais processos apresentam desafios significativos, como a regulação de genes e metabólitos, além da necessidade de otimização e escalonamento para níveis industriais. Entre as principais consequências negativas, destacam-se a inibição do crescimento e da viabilidade celular devido

à sobrecarga metabólica imposta ao microrganismo, o que resulta em redução da produtividade (Rasor et al., 2021).

Por outro lado, os processos de produção de metabólitos e extratos fermentados livres de células operam fora das restrições impostas pelas células vivas, sendo viabilizados pelo uso de extratos celulares brutos ou enzimas purificadas (Claassens et al., 2019). Essas abordagens são amplamente aplicadas na catálise de reações in vitro e apresentam vantagens relevantes, como a eliminação da necessidade de manutenção da viabilidade celular, a possibilidade de modular vias metabólicas sintéticas e o controle direto e preciso das condições reacionais, incluindo pH, temperatura e concentrações de substratos e cofatores (Sanchez & Demain, 2011; Sheldon & Woodley, 2018).

Apesar dessas vantagens, o uso de extratos livres de células também enfrenta desafios, entre eles a padronização, estabilidade e robustez do processo, bem como os custos associados à purificação dos componentes, quando necessário. Diversos estudos têm sido conduzidos visando o desenvolvimento de protocolos que otimizem os processos e aumentem a estabilidade desses extratos em aplicações industriais (Dudley et al., 2015; Rasor et al., 2021).

De modo geral, os bioprocessos industriais ainda utilizam predominantemente células viáveis, especialmente em operações de larga escala, devido ao melhor custo-benefício dessas tecnologias. Em contrapartida, os sistemas livres de células vêm se consolidando como ferramentas complementares de grande potencial. Eles se destacam em aplicações que demandam rápida concepção e testes, elevada flexibilidade e controle preciso, ou na produção de compostos tóxicos para células vivas (Claassens et al., 2019; Ekas et al., 2019; Sheldon & Woodley, 2018). Com o avanço contínuo dessas técnicas, espera-se que se tornem cada vez mais viáveis e menos onerosas, ampliando seu uso em setores como agricultura, indústria alimentícia, saúde e meio ambiente.

2.5. Biodegradação

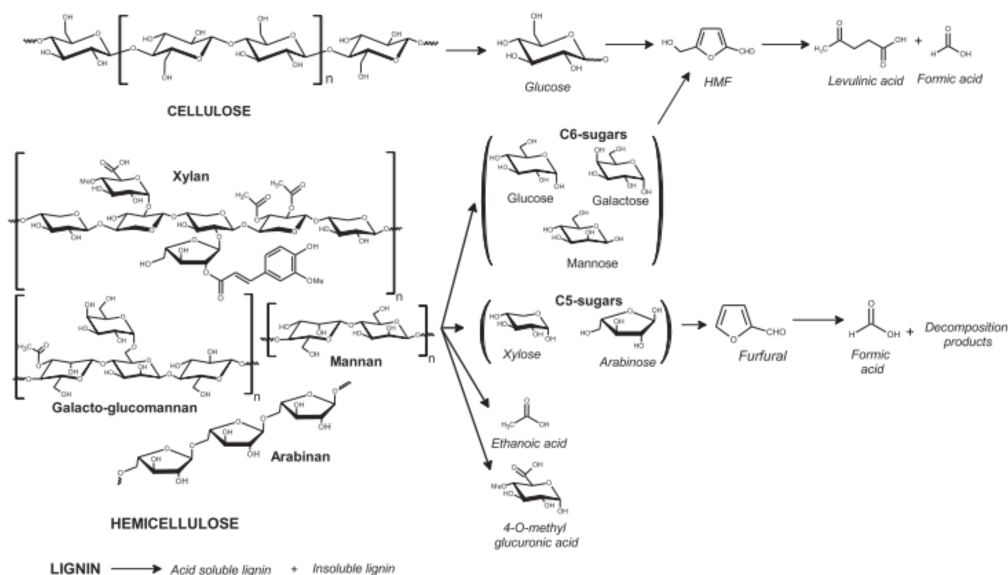
2.5.1. Biodegradação da biomassa lignocelulósica

A biomassa lignocelulósica é a fonte de carbono renovável mais abundante no mundo. Composta majoritariamente por celulose, hemicelulose e lignina, estes compostos podem ser hidrolisados em açúcares e, então, processados a fim de terem um valor agregado. Uma das maneiras de se agregar valor a estes resíduos é a utilização destes como substrato para produção

de enzimas através da fermentação (Behera & Ray, 2016). A fermentação é a principal via responsável pela conversão da biomassa lignocelulósica por microrganismos. Dependendo do tipo de fermentação e as condições metabólicas do microrganismo, é possível degradar a biomassa e sintetizar compostos de interesse simultaneamente, como é o caso das enzimas, tornando-se uma opção interessante a ser utilizada nas indústrias (Datta et al., 2020).

Durante o processo fermentativo, ao entrar em contato com o resíduo, o microrganismo sinaliza a presença de macromoléculas ao seu redor e sintetiza a enzima de interesse. Após a sinalização, os polímeros são degradados em monômeros e, então, estes passarão a atuar como principal fonte de energia e carbono para o crescimento microbiano. O produto final da biodegradação polimérica geralmente resulta em biomassa microbiana, H_2O e CO_2 (Hu et al., 2023). Dentre estes polímeros, a lignina apresenta a estrutura mais complexa, contendo anéis aromáticos interligados por diferentes ligações covalentes de C – C e C – O que conferem à molécula uma característica hidrofóbica e uma estrutura recalcitrante de difícil acesso (Sun et al., 2018). A conversão da biomassa lignocelulósica segue um caminho complexo, principalmente a nível molecular, uma vez que envolve vários produtos intermediários, conforme mostra a Figura 14.

Figura 14 - representação esquemática da degradação das frações poliméricas presentes na biomassa lignocelulósica



Fonte: Girisuta et al. (2013)

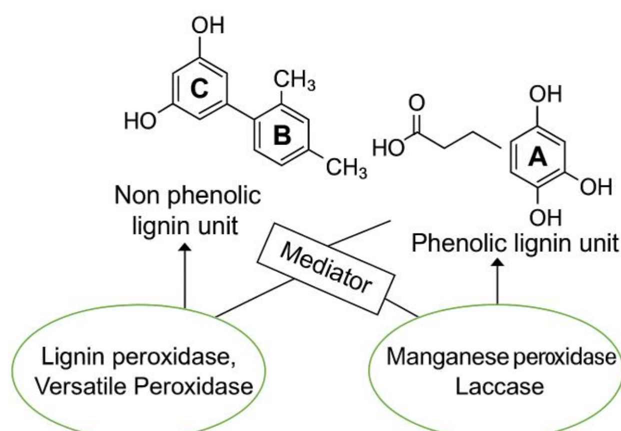
As enzimas capazes de oxidar estas estruturas complexas são as ligninases (lacase, manganês peroxidase, peroxidase versátil e lignina peroxidase). Dentre as ligninases, destaca-se a lacase, sendo a enzima que possui seus mecanismos de degradação mais elucidados e

estudados. Os fatores que mais afetam a síntese enzimática são a temperatura, a composição do meio de cultura e a presença ou ausência de mediadores fenólicos na reação. O foco da degradação pela lacase é nos compostos aromáticos presentes na lignina, contudo, seu ataque é limitado, uma vez que sua estrutura não é representada somente pelos anéis fenólicos (Elegir et al., 2008; Nigam et al., 2018).

Ainda não existem estudos que mostrem a conversão completa da biomassa lignocelulósica através de enzimas microbianas. Além disso, os mecanismos de degradação biológica da lignina são pouco elucidados, uma vez que são gerados muitos metabólitos e substâncias alifáticas e os mecanismos de ação ainda são complexos. Deve-se considerar também que as espécies e as técnicas utilizadas para síntese enzimática podem apresentar diferentes resultados e, portanto, não é algo padronizado (S. Khatami et al., 2019; Yada et al., 2019).

Geralmente, os estudos relacionam a produção enzimática com a degradação dos polímeros presentes na biomassa lignocelulósica. Como existem enzimas específicas para atuar em cada molécula, possivelmente a síntese de ligninases implicará na degradação de lignina, seu principal alvo. Para caracterizar e quantificar a degradação de lignina, utiliza-se a Cromatografia Líquida de Alta Eficiência, que é capaz de identificar os compostos de degradação (Datta et al., 2020; de Carvalho Oliveira et al., 2018).

Figura 15 - Representação esquemática do alvo de cada ligninase, onde é possível visualizar o potencial de ação da lignina peroxidase e peroxidase versátil sobre as frações não fenólicas e manganês peroxidase e lacase sobre as frações fenólicas da lignina



Fonte: Elaborado pelo autor (2025)

2.5.2. Biodegradação da atrazina

A biorremediação constitui uma abordagem fundamental para a remoção de poluentes orgânicos no ambiente, a qual pode ser definida como um "processo biologicamente mediado durante o qual um composto indesejado é transformado, degradado, sequestrado e/ou totalmente removido" (Vaksmaa et al., 2023). Esse processo pode ocorrer em duas modalidades: ex situ e in situ (V. Singh et al., 2024).

As enzimas são essenciais para que esse processo ocorra, facilitando as reações bioquímicas, diminuindo a energia de ativação necessária e estabilizando o estado de transição (Khan, 2021). Nesse sentido, as enzimas exibem especificidade por meio de seus sítios ativos, onde os substratos se ligam. Dentre elas, as oxidorredutases têm recebido atenção especial por sua aplicação em biorremediação (Mousavi et al., 2021b).

A degradação de atrazina e desetilatrazina, consideradas poluentes orgânicos, por enzimas pode ocorrer por meio de dois mecanismos distintos: atividade metabólica e atividade cometabólica. A atividade metabólica denota um cenário em que os poluentes atuam como substrato primário de crescimento para um organismo, estimulando a produção enzimática visando sua degradação (S. Singh et al., 2024). Por outro lado, o cometabolismo surge quando um organismo sintetiza enzimas para degradar um substrato primário de crescimento alternativo. Essas enzimas também podem atuar sobre compostos estruturalmente análogos, embora o organismo não os utilize para seu crescimento (V. Singh et al., 2024).

Tanto em vias metabólicas, quanto em vias cometabólicas, a atrazina é degradada em uma variedade de produtos: hidroxiatrazina (HA), desetilatrazina (DEA) e deetilhidroxiatrazina (DEHA), desisopropilatrazina (DIA) e desetildeseopropilatrazina (DEDIA), ácido cianúrico, isopropilamina, biureto e alofanato. Todos os subprodutos resultantes da degradação são similares a atrazina, sendo considerados tóxicos. Um dos principais motivos que mantêm a toxicidade destes compostos – mesmo após a degradação – é a capacidade de persistência e consequente contaminação do ambiente (S. Singh et al., 2024).

As enzimas aptas à degradação cometabólica apresentam uma versatilidade considerável. Quando as características estruturais desses poluentes se assemelham às do substrato primário de crescimento, é visto que as respectivas enzimas degradam efetivamente esses poluentes (Kennes-Veiga et al., 2022a). No entanto, a relação entre a estrutura da atrazina

e da desetilatrazina, bem como a capacidade de degradação enzimática permanece incompreendida.

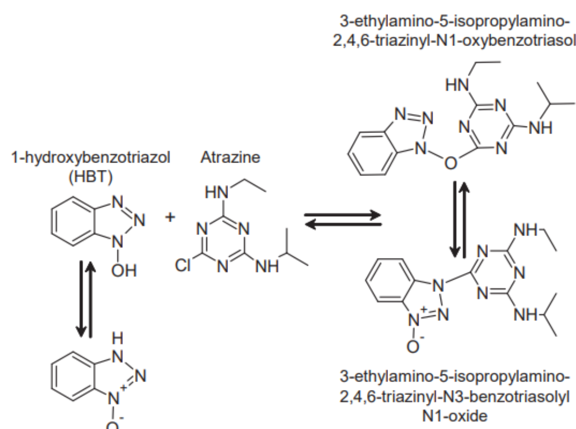
Além disso, não são apenas as propriedades estruturais de poluentes que influenciam a atividade enzimática; por exemplo, Tran et al. (2013) observaram que o diclofenaco, um poluente orgânico, apresentou baixa remoção por enzimas derivadas de consórcio bacteriano, mas foi significativamente degradado por enzimas análogas derivadas de fungos. Essa diferença ressalta a influência do organismo produtor na funcionalidade e interação da enzima com o poluente, afetando seu potencial de degradação.

As oxidorredutases apresentam a capacidade de facilitar a remoção de poluentes por meio de uma infinidade de mecanismos, estabelecendo uma ampla gama de enzimas candidatas à degradação desses poluentes (Mousavi et al., 2021b). Apesar da grande variedade, ainda há uma compreensão limitada acerca dos mecanismos enzimáticos envolvidos no processo (Kennes-Veiga et al., 2022a).

Entre as oxidorredutases frequentemente empregadas em biorremediação estão as lacases e diversas peroxidases (Nawaz et al., 2024). Embora a maior parte das pesquisas tenham se concentrado nas lacases produzidas por fungos, essas enzimas também podem ser sintetizadas por algumas bactérias. Por outro lado, as enzimas peroxidases são mais amplas, produzidas amplamente por fungos e bactérias (Aza & Camarero, 2023; Sellami et al., 2022). Dito isso, a ação combinada das enzimas lacase, lignina peroxidase e manganês peroxidase pode ser fundamental na degradação de compostos orgânicos complexos.

A degradação de herbicidas, inclusive a atrazina, por lacases é reconhecida (Jia et al., 2024). Embora pouco elucidados, existem sistemas reacionais nos quais, com a presença de lacase e atrazina, um subproduto (HBT: hidroxibenzotriazol) é formado e, sob ação catalítica, o herbicida é degradado em produtos menos tóxicos, conforme representado pela Figura 16.

Figura 16 - Rota metabólica da degradação da atrazina mediado por lacase e hidroxibenzotriazol



Fonte: Koroleva et al. (2015)

2.6. Alinhamento do trabalho à agenda 2030 da ONU

Ações governamentais tendem a desenvolver a economia circular através do desenvolvimento sustentável. A Organização das Nações Unidas (ONU) propôs um plano onde são destacados 17 Objetivos de Desenvolvimento Sustentável (ODS), também conhecidos como Objetivos Globais como um chamado universal à ação para erradicar a pobreza, proteger o planeta e garantir que, até 2030, todas as pessoas desfrutem de paz e prosperidade. A principal finalidade é garantir que as próximas gerações tenham qualidade de vida bem como promover o bem-estar por meio do acesso à comida e água de qualidade, além de objetivos sociais como educação e inclusão (*Objetivos de Desenvolvimento Sustentável | As Nações Unidas No Brasil*, n.d.). Os 17 ODS são integrados, uma vez que reconhecem que a ação em uma área afetará os resultados em outras e que o desenvolvimento deve equilibrar a sustentabilidade social, econômica e ambiental.

Este trabalho está diretamente está alinhado aos Objetivos de Desenvolvimento Sustentável (ODS) da Agenda 2030 da ONU, sobretudo ODS 2, ODS 6, ODS 12, ODS 13 e ODS 17.

ODS 2 - Fome Zero e Agricultura Sustentável: os objetivos desse trabalho visam melhorar o crescimento e a produtividade das plantas por meio do desenvolvimento de um extrato bruto enzimático derivado da cana de açúcar. Este extrato que pode aumentar a eficiência da agricultura ao se provar eficiente no manejo de pragas e contribuir para a segurança alimentar, sem a necessidade de insumos químicos sintéticos. Além disso, pode se provar eficiente ao degradar contaminantes emergentes, contribuindo diretamente para a remediação de solo e águas contaminados.

ODS 6 - Água potável e saneamento: os objetivos deste trabalho visam garantir a gestão sustentável da água por meio do desenvolvimento de alternativas para remediação de contaminantes emergentes, comumente encontrados na água e no solo.

ODS 12 - Consumo e Produção Responsáveis: nota-se que ao utilizar resíduos de cana de açúcar para produção de extratos fermentados, busca-se minimizar o desperdício e transformar resíduos em subprodutos ou produtos de valor agregado, promovendo uma economia circular e uma gestão eficiente dos recursos naturais.

O ODS 13 - Ação contra a Mudança Global do Clima: apoia práticas agrícolas sustentáveis que podem contribuir para a mitigação dos efeitos das mudanças climáticas por

meio da promoção da economia circular e redução da pegada de carbono. O uso dos resíduos da cana de açúcar para formulação de extratos fermentados incentiva a agricultura de baixo impacto ambiental e reduz a dependência de insumos químicos, contribuindo para a redução das emissões de gases de efeito estufa e aumentando o sequestro de carbono por meio da promoção de uma maior atividade microbiana no solo.

Por fim, o projeto abriga o ODS 17 - Parcerias e Meios de Implementação: pois integra ciência, tecnologia e práticas agrícolas, através do fomento de parcerias entre a academia, a indústria e outros setores, possibilitando a introdução de uma nova patente acerca do extrato fermentado obtido.

SEGUNDA PARTE - ARTIGOS

ARTIGO 1 – Optimization of cell wall-degrading enzymes production by *Penicillium brevicompactum* and effects on sugarcane bagasse

Redigido conforme norma do periódico científico - Versão preliminar	
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Periódico:	Science of The Total Environment (IF: 8.2)
ISSN	1981-4747

Abstract

Lignocellulosic waste is produced during the processing of various agricultural products. To prevent the accumulation of this waste in unsuitable areas, it is essential to convert it into value-added products. The production of enzymes from lignocellulosic biomass is an area of active research; however, high production costs and the selection of suitable strains continue to pose challenges. This study focused on optimizing the production of cell-wall degrading enzymes (CWDEs) using a new strain, *Penicillium brevicompactum*, using sugarcane bagasse as the substrate and primary carbon source. We achieved maximum enzyme activity for all tested enzymes, including carboxymethylcellulase (CMCase), total cellulase (FPase), xylanase, pectinase, avicelase, and β-glucosidase, at a moisture content of 65% after 7 days of fermentation. Modifications in the sugarcane bagasse (SCB) were analyzed using scanning electron microscopy, Fourier-transform infrared spectroscopy, and high-performance liquid chromatography. Response surface methodology proved significant for four out of the six response variables: pectinase, FPase, CMCase, and xylanase. The SCB exhibited structural and chemical composition changes when compared to the control group (no fermentation). Our results suggest that *P. brevicompactum* could provide a more feasible and cost-effective method for enzymatic production through solid-state fermentation. These findings may contribute to the future advancement of enzyme production and biomass conversion for practical applications.

Keywords: cell-wall degrading enzymes; biomass conversion; central composite rotational design; *Penicillium brevicompactum*.

1. Introduction

Tons of lignocellulosic waste are generated every year, especially in countries where agribusiness is the leading country's gross domestic product (GDP) (Mujtaba et al., 2023). However, agro-industrial waste mismanagement leads to accumulated biomass in locals that is inappropriately disposed of (Rai & Kundu, 2024). Sugarcane is an important crop, low-costly produced, and mainly used for foods, beverages, and alternative ethanol biofuel production (Batool et al., 2025). The 2024/25 crop is expected to see the world's sugarcane production surpass 640 billion tonnes, with Brazil leading the production (CEPEA, 2024). The amount of lignocellulosic feedstock from bagasse has drawn attention as a promising alternative for enhancing the production and energy optimization of the ethanol-obtaining process, primarily by the secondary generation of ethanol, as well as other value-added applications (Candido & Gonçalves, 2019; Torgbo et al., 2021). Understanding their chemical composition enables its exploitation for biotechnological applications.

Fermentation, particularly through enzymatic hydrolysis, is essential for transforming these residues into valuable products. Thus, one approach is using these residues as a substrate for enzyme production through solid-state fermentation (SSF). This process allows microorganisms to degrade biomass and synthesize valuable compounds, such as enzymes, simultaneously, making it a promising option for industrial applications (Pandey et al., 2000). Several enzymes can be produced during this process, with the culture conditions and fermentation parameters one can favor others. However, when using lignocellulosic biomass, it is expected that plant cell-wall degrading enzymes will be produced. The main enzymes to be produced are cellulases, hemicellulases, ligninases, and pectinases (Leite et al., 2021). Using the appropriate species in this process yields better results. For instance, *Penicillium brevicompactum* is a significant species with biotechnological applications, particularly in the production of metabolites (Brown et al., 1976; Regueira et al., 2011).

Recently, great attention has been paid to the direct application of cell-wall degrading enzymes (CWDE's). They develop a crucial role in major applications. For example, cellulases (EC 3.2.1.203; EC 3.2.1.91; EC 3.2.1.21) play a critical role in composting, enhance biofertilizers, and improve the digestibility of silage (Giovannoni et al., 2020). Hemicellulase (EC 3.2.1.8) can enrich both soil and animal feed. Pectinase (EC 3.2.1.15) processes plant extracts, softens seed coats, and promotes soil health by breaking down pectins found in plant residues (Andlar et al., 2018). Not only enzymes, but several biobased products can be produced during SSF, such as surfactants, biopesticides, antibiotics, among others (Rocha et al., 2024).

Despite the recognition of these applications, high production costs associated with low activity and stability of available purified enzymes or other metabolites restrict their commercial use (Klein-Marcuschamer et al., 2012). The production of a suitable SSF biological product depends on efficient microbial growth and enzymatic production, which is influenced by fermentative conditions. It is known that a series of parameters influence SSF, such as temperature, initial moisture content, incubation time, aeration, and nutrient availability in the substrate (Šelo et al., 2021; Zadrazil & Puniya, 1995). However, there is still a lack of studies on optimal operating conditions. In this sense, this study investigates: (i) the optimal conditions for the production of cellulases, xylanase, and pectinase by a new producing species; (ii) the chemical and morphological characterization of the SCB used as substrate for the fermentation; (iii) the determination of target SCB composition compounds during fermentation.

2. Materials and methods

2.1 Culture conditions and substrate preparation

The *Penicillium brevicompactum* strain was isolated from Amazonian soil and available from the Culture Collection of the Department of Food Science of the Federal University of Lavras (UFLA), Minas Gerais, Brazil.

Penicillium brevicompactum was cultured in Malt Extract Agar medium (MEA), with the following composition (g/L): glucose (15), malt extract (15), and agar (15) and incubated at 25 °C for seven days. The inoculum was prepared by adding 10 mL of 0,1% Tween 80 to the fungal colony and spread using a Drigalski loop. Conidia counting was performed in a Neubauer chamber and standardized to a final count of 1.5×10^8 conidia/g.

The SCB was provided by Cachaçaria Carinhosa, located in Perdões, Minas Gerais (21°4’4.058” S 44°59’29.036” W). The samples were dried in an oven for 72 h, until they reached a constant weight. Then, the SCB was ground into 1.0-mm particles (18 mesh), stored in impermeable polypropylene plastic bags and kept at room temperature.

2.1.1 Optimization of enzyme production through a rotational central composite design (CCRD)

The optimization of cell-wall degrading enzyme production was carried out using the central composite rotational design (CCRD) experimental framework, focusing on time (days) and moisture percentage (%) as independent variables. These factors were chosen for their significance in solid-state fermentation and enzyme production (A. T. da Silva et al., 2025). The impact of these variables on the response variable, which is the production of cell-wall degrading enzymes, was analyzed at five experimental levels: time intervals of 24, 21, 14 (the central point), 7, and 4 days, and moisture levels of 63, 65, 70 (the central point), 75, and 77%. A total of thirteen experiments were conducted, including five replications at the central point, as detailed in Table 1.

Table 1: Experimental design matrix, considering two variables (time and moisture) and five repetitions at the central point, totaling 13 tests

Run	Time (days)	Moisture (%)
1	7	65
2	21	65
3	7	75
4	21	75
5	4	70
6	24	70
7	14	63
8	14	77
9	14	70
10	14	70
11	14	70
12	14	70
13	14	70

Solid-state fermentation (SSF) was performed in 250-mL Erlenmeyer flasks containing 15 g of sterilized SCB at 23 °C according with the methodology proposed by de Souza Castro et al. (2024). The enzyme production media consisted of the following composition (g/L): sodium nitrate (9), monopotassium phosphate (2.25), potassium chloride (0.75), magnesium sulfate (0.75), iron sulfate (0.015), and zinc sulfate (0.015). The media was buffered with citrate-phosphate (0,1 M) solution at pH 5.0 and inoculated with the conidia suspension (1.5×10^8 conidia/g). Homogenization was performed with a sterile glass rod.

To obtain the crude extract, 75 mL of distilled water (in a ratio of 1:5) were added to each Erlenmeyer flask. The flasks were kept under constant agitation at 120 rpm for 60 minutes in an ice bath. Then, the samples were filtered using voile fabric, centrifuged at 11,180 xg for 15 minutes, transferred to Falcon tubes and stored in a freezer at -22 °C until quantification of enzymatic activity (Soares et al., 2012).

The model was validated by repeating three of the thirteen preconditions in the matrix under the same experimental conditions.

2.2 Enzyme assays

Xylanase (EC 3.2.1.8) was assayed using 1% wood xylan as the substrate for 10 minutes at 50 °C, pH 4.8 (Nunes et al., 2024b). One enzyme unit was defined as the amount of xylanase required to release 1 mg of xylose per minute under the assay conditions.

Carboxymethylcellulase (CMCase: EC 3.2.1.203) and avicelase (EC 3.2.1.91) were assayed using carboxymethylcellulose and avicel as the substrate, respectively, for 30 minutes at 50 °C, pH 5.0 (Prasanna et al., 2016; Salazar et al., 2020). Total cellulase (FPase) was assayed using Whatman n°1 filter paper as the substrate for 60 minutes at 50 °C, pH 5.0 (Ghose, 1987). One enzyme unit was defined as the amount of CMCase, avicelase, and FPase required to release 1 μmol of glucose per minute under the assay conditions.

β-glucosidase (EC 3.2.1.21) was assayed using 4 mM p-nitrophenyl-D-glucopyranoside as the substrate for 30 minutes at 50 °C, pH 5.0 (Santa-Rosa et al., 2018). One enzyme unit was defined as the amount of β-glucosidase required to release 1 mg of *p*-nitrophenol per minute under the assay conditions.

Pectinase (EC 3.2.1.15) was assayed using 0.2% pectin as the substrate for 25 minutes at 35 °C, pH 4.5 (Ahmed et al., 2016). One enzyme unit was defined as the amount of pectinase required to release 1 mg of galacturonic acid per minute under the assay conditions.

All the assays were read at 540 nm.

2.3 SCB chemical characterization

2.3.1 Chemical composition using gravimetric analysis

Lignin was estimated according to the modified Klason lignin determination procedure by using the extractive-free sample, using 72% sulfuric acid. Holocellulose from extractive-

free sample was estimated by using 30% sodium chlorite and 20% acetic acid. The cellulose content in holocellulose was determined using 24% sodium hydroxide. All three components were determined based on the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2008).

2.3.2 High Performance Liquid Chromatography (HPLC)

Total oligomer hydrolysis was performed based on Gouveia et al. (2009). All hydrolyzed samples were extracted using SPE cartridges Sep-Pak C18 (Waters) to avoid phenolic and other interferents. The compounds glucose, cellobiose, formic acid, and hydroxymethylfurfural were considered the cellulose fraction, and xylose, arabinose, glucuronic acid, acetic acid, and furfural were considered the hemicellulose fraction. All analyses were conducted using the same model of liquid chromatography (LC-10Ai; Shimadzu Corp., Tokyo, Japan) equipped with a dual detection system, comprising a UV-vis detector set to 210 nm (SPD-10Ai; Shimadzu) and a refractive index detector (RID-10Ai; Shimadzu).

For the determination of carbohydrates and organic acids, a Shimadzu ion exclusion column (Shim-pack SCR 101-H, 7.9 mm × 30 cm) was employed, using ultrapure water acidified with $HClO_4$ (pH 2.1) as the mobile phase, at a flow rate of 0.6 mL/min and an oven temperature of 50 °C. Concentrations were established from calibration curves of the pure compounds, which included glucose, cellobiose, xylose, arabinose, formic acid, glucuronic acid, and acetic acid.

The analysis of furfural and hydroxymethylfurfural was similarly performed using a liquid chromatograph with a dual detection system, featuring a UV-vis detector set to 276 nm (SPD-10Ai; Shimadzu), a refractive index detector (RID-10Ai; Shimadzu), and a C18 column (LC18, 15 cm × 4.6 cm). The mobile phase consisted of acetonitrile and water at a 1:8 (v/v) ratio with 1% acetic acid, flowing at a rate of 0.8 mL/min at a temperature of 27 °C, while the UV detector was set to 276 nm. Concentrations for furfural and hydroxymethylfurfural were likewise determined from calibration curves of the pure compounds.

2.4 Scanning electron microscopy (SEM)

The samples from both the fermentation and control groups were dried after the enzymes were extracted, then fixed to an aluminum support using double-sided carbon tape. They were subsequently coated with a gold layer of approximately 20 nm using a Sputter Coater. The

images were observed in a Scanning Electron Microscope (SEM) at an acceleration voltage of 20 kV and a working distance of about 38 mm (Moretti et al., 2016). Hundreds of SEM images were obtained from different areas of the samples to ensure the reproducibility of the results.

2.5 Fourier transformed infrared spectroscopy (FTIR)

For the infrared analyses, an IRAffinity-1 FTIR spectrophotometer (Shimadzu, Kyoto, Japan) was utilized. The analysis was conducted over a spectral range of 400 to 4000 cm^{-1}, using 64 scans at a resolution of 2 cm^{-1}. The samples from both the fermentation and control groups were dried after the enzymes were extracted, and then they were prepared by means of a KBr disk containing 3% finely ground samples (Liu et al., 2014).

2.6 Data analysis

The optimization matrix, along with all analyses—including response surface analysis, ANOVA, f-test, and contour plots—was created using R Statistical Software (version 2024.12.1; R Core Team, 2021). The FTIR graph images and plots were generated using OriginPro Student Version 2023 (OriginLab Corporation, Northampton, MA, USA). The SCB characterization data was normalized to a 0-1 Min-Max scale, and ANOVA and t-tests were conducted and plotted using R.

3. Results and discussion

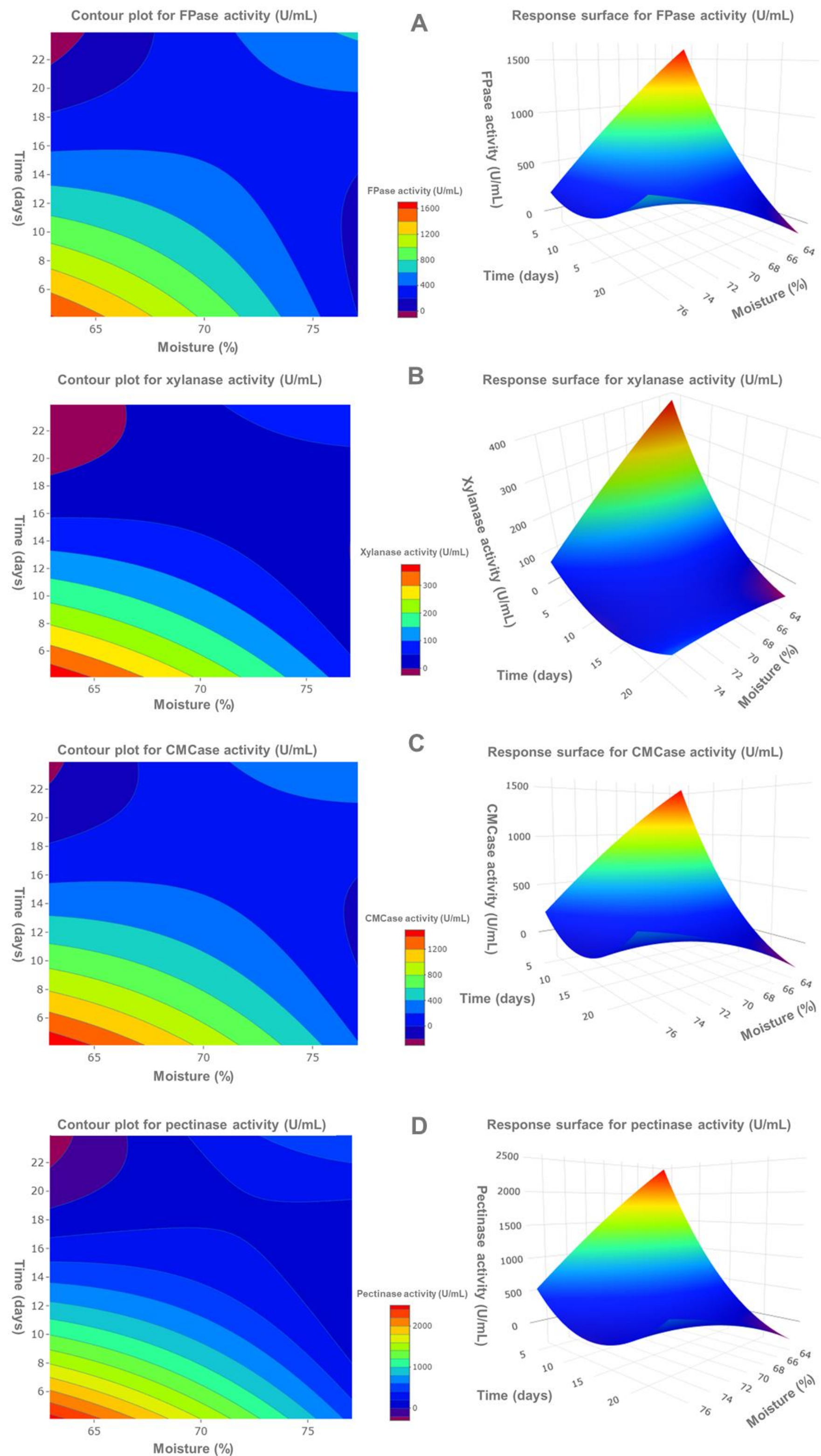
3.1 Enzyme optimization

A total of 13 runs with different times and moisture content were performed. After excluding terms related to non-significant variables ($p < 0.05$), the equation for the final response (enzyme activity) is presented in Table 2. The F test confirmed the significance of the regression model statistics, and analysis of variance (ANOVA) was employed for the quadratic response surface model. Table 2 shows that the regression coefficients underscored the significance of the model, yielding an R^2 value of > 0.82 for the significant variables. Both avicelase and β-glucosidase exhibited limited activity under the tested conditions, with values of 5.4 and 5 U/mL, respectively; however, the regression models indicated no significance.

Response surface plots for all the enzymes were constructed despite their significant interactions that showed a positive effect on enzyme production. They are presented in Figure

1. Four out of the six-dimensional graphs (Fig. 1A, B, C, D) showed a significant effect on interactions between these parameters on CWDE’s production.

Figure 17: Response surface and contour plots for the activity of all significant models: pectinase, carboxymethylcellulase, FPase, and xylanase, under two different variables: time and moisture content.



Legend: A – response surface and contour plots for FPase; B response surface and contour plots for xylanase; C – response surface and contour plots for CMCase; D - response surface and contour plots for pectinase.

Table 2: Final response equations from the surface response design and each enzyme's R^2 and activity (U/mL) at the optimal conditions (65% (w/w) moisture content and 7 days of fermentation).

Enzymes	Final response equation	R^2	Activity
FPase	$Y = 4468.91 + 41{,}63X_2 + 7{,}7075X_1X_2$	0.82	1359.4
Pectinase	$Y = 6287.39 - 935{,}95X_1 + 69X_2 + 10{,}15X_1X_2 + 5{,}60X_1^2 - 1{,}77X_2^2$	0.88	1786.2
CMCase	$Y = 2458.09 - 625{,}82X_1 + 94{,}21X_2 + 7{,}01X_1X_2 + 3{,}35X_1^2 - 1{,}55X_2^2$	0.91	1127.8
Xylanase	$Y = 1127.05 - 149{,}18X_1 + 7{,}21X_2 + 1{,}63X_1X_2 + 0{,}87X_1^2 - 0{,}25X_2^2$	0.93	284.4

Legend: Y – enzyme activity; X_1 – coded level for time; X_2 – coded level for moisture content.

The SCB fermentation took place with an inoculum concentration of $1.5x10^8$ conidia/g, a pH of 5.0, a temperature of 23 °C, and aeration conditions established based on existing literature. Two important parameters in solid-state fermentation are moisture content and incubation time. The highest enzyme activities were observed at a moisture level of 65% (w/w), with an incubation period of 7 days for all the enzymes. The model indicates that a combination of incubation time and lower moisture content, under the pre-selected conditions, positively influences the production of cell-wall-degrading enzymes (CWDEs) such as FPase, pectinase, carboxymethylcellulase, and xylanase. Although it was not significant for avicelase and β-glucosidase, we still were able to identify their production.

The model was validated, and three pre-established experimental conditions from the matrix were repeated: 14 days at 70% moisture content, 4 days at 70% moisture, and 7 days at 65% moisture. The results were consistent with our observations from the experimental conditions, showing variations of approximately 5% for all the tested enzymes.

Detecting and optimizing the production of CWDEs from new species is crucial due to their significant industrial applications. Cellulases and hemicellulases convert lignocellulosic biomass into fermentable sugars for second-generation ethanol, thereby enhancing sustainability by utilizing agro-industrial waste (Mansuri & Shekhawat, 2024). In the food industry, these enzymes clarify juices and wines and improve oil extraction from plants(Bhattacharya et al., 2024) . They also enhance pulp quality in paper production and soften textiles. Additionally, they facilitate protoplast production for genetic studies and aid in extracting valuable compounds while promoting the biodegradation of lignocellulosic waste (Goda et al., 2024).

Penicillium species are well-known for their ability to produce hydrolytic enzymes, such as cellulases, xylanases, and pectinases (Huang et al., 2024). This enzyme production is driven by specific genes that code for these enzymes, as well as the ecological roles these fungi play in various environments. Most *Penicillium* spp. are classified as either saprophytic or phytopathogenic, which allows them to express these genes under specific conditions (Rozhkova

et al., 2022). It is important to note that *Penicillium* belongs to the Ascomycete group, and these species typically utilize simpler substrates to produce their enzymes (Shankar et al., 2023). In contrast, species from the Basidiomycete group generally thrive on more complex substrates, such as lignin (Ma et al., 2021). Our strain, however, has the capability to utilize a complex substrate like sugarcane bagasse (SCB) to produce several enzymes.

Using SCB as a substrate for fermentation promotes fungal growth and simulates natural conditions, allowing for the observation of mycelial growth on the surface of SCB particles during the incubation period. Additionally, SCB enhances gene expression in these circumstances and contributes to the production of lignocellulolytic enzymes. As a complex substrate, it simultaneously induces the production of all cell wall-degrading enzymes (CWDEs) essential for cell wall breakdown, producing lignocellulolytic enzymes (Asemoloye et al., 2021). In this sense, each of these enzymes' synergist action promotes a homogeneous lignocellulose biomass hydrolysis. However, it also depends on the type of substrate, the fungal physiology, and the genetic apparatus (Saini et al., 2022). To the best of our knowledge, this is the first report on the production of CWDEs from *P. brevicompactum.* Thus, it is important to highlight the production of each enzyme.

Pectinase was found to be the most abundant enzyme, exhibiting an activity level of 1786.2 U/L. The production of cellulase was assessed using a variety of substrates: filter paper, avicel, carboxymethylcellulose, and p-nitrophenyl-D-glucopyranoside. The respective enzyme activities (in U/mL) were as follows: 1359.4 for filter paper, 5.4 for avicel, 1127.8 for carboxymethylcellulose, and 5.0 for p-nitrophenyl-D-glucopyranoside. The highest recorded xylanase activity was 284.4 U/mL.

Chavan et al. (2024) optimized the culture conditions for *Penicillium funiculosum* to increase cellulase production. They achieved elevated enzyme activities, including 65 FPU U/mL for FPAse, 65 U/mL for β-glucosidase, 55 U/mL for CMCase, and 40 U/mL for xylanase over a six-day fermentation period using avicel and wheat bran as inducers at a temperature of 30 °C. Conversely, Núñez-Serrano et al. (2024) focused on optimizing culture conditions for *Penicillium crustosum* to maximize the production of pectinase, xylanase, and endoglucanase (avicelase) using lemon peel and wheat bran as substrates. When lemon peel was utilized, they obtained higher enzyme activities for pectinase (971.5 U/mL) and endoglucanase (56.2 U/mL), though xylanase activity was lower at 47.2 U/mL. In contrast, using wheat bran, they achieved a xylanase activity of 236.3 U/mL.

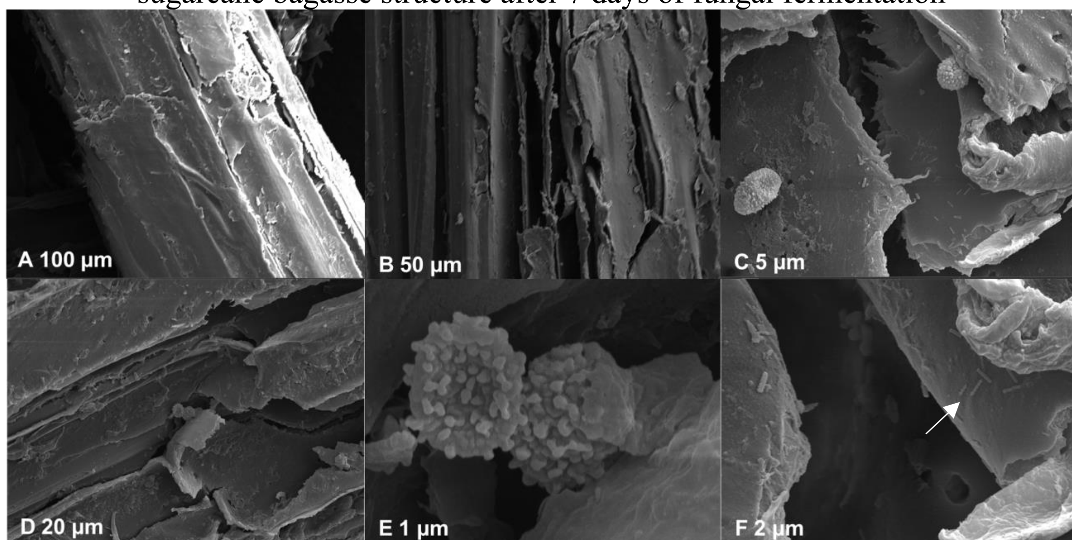
Our findings suggest that *P. brevicompactum* could be an excellent source of cell wall-degrading enzymes (CWDEs), as indicated by the higher enzyme activities observed in our study

compared to other research, with the exception of β-glucosidase and avicelase. This implies that further optimization of parameters may be necessary to enhance the activities of these specific enzymes.

3.2 Chemical and morphological characterization of sugarcane bagasse

To evaluate the effect of the fungal fermentation on the morphology of SCB biomass to detect enzymatic degradation, we used scanning electron microscopy to investigate the morphological changes that occurred during fermentation. Figure 2 shows the effects of fermentation on sugarcane bagasse, compared to raw material (no fermentation).

Figure 2: Scanning electron microscopy (SEM) to evaluate morphological changes on the sugarcane bagasse structure after 7 days of fungal fermentation



Legend: A – SCB surface without modifications and no fermentation; B – SCB surface after 7 days of fungal fermentation; C – fungal spores adhered in cellulose walls after 7 days of fermentation; D – SCB cellulose layers with modifications after 7 days of fermentation; E – fungal spores during the colonization of the biomass; F – cellulose nanocrystals.

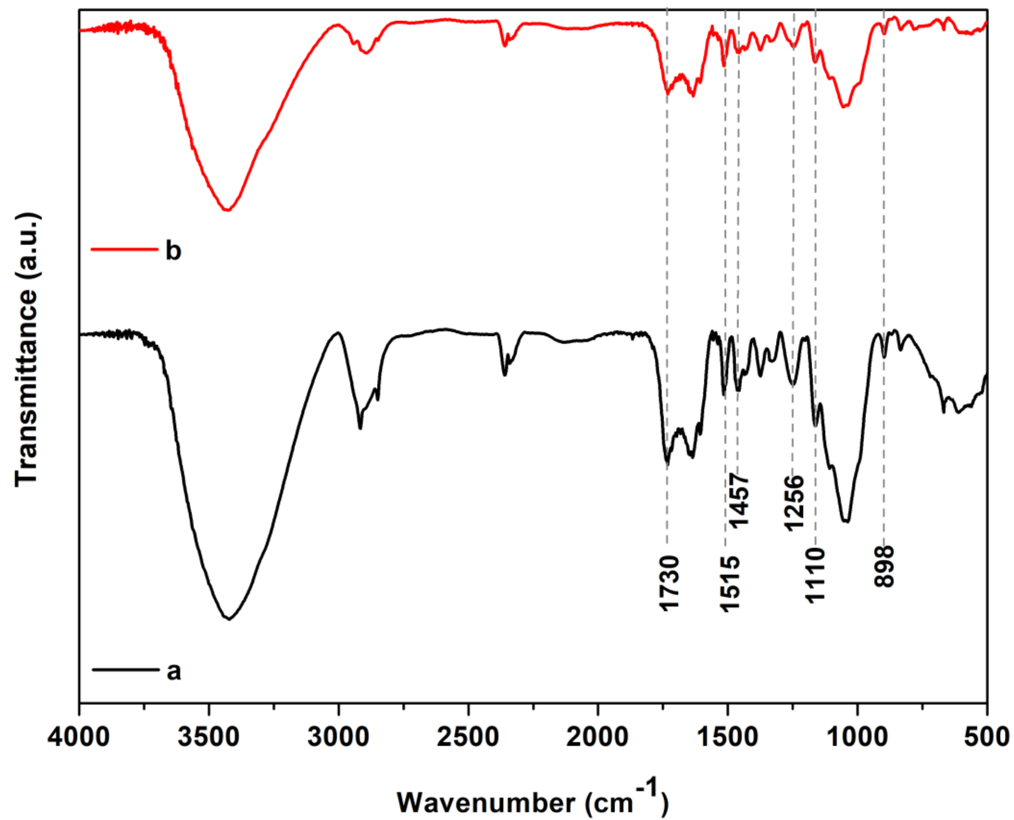
Figure 2A shows the surface of untreated sugarcane bagasse, highlighting its intact fibrous structure, well-organized cell walls, and the absence of degradation or microbial colonization. After 7 days of fungal fermentation, as illustrated in Figure 2B, the biomass undergoes significant destructuring. The enzymatic action of the fungus increases the porosity and fragmentation of the fibers, indicating partial degradation of cellulose and hemicellulose. In Figure 2C, the presence of the microorganism in the biomass is confirmed, revealing fungal spores adhered to the cellulose cell walls. This adhesion suggests an active colonization process that contributes to the degradation of lignocellulosic materials. The degradation becomes even

more evident in Figure 2D, where the cellulose layers exhibit signs of structural modification, characterized by cracks and fragmentation of the fibers. This reinforces the effectiveness of the fungal enzymes on the polymer matrix of the biomass. Figure 2E highlights fungal spores at various stages of development during their colonization of the substrate. The germination of these spores indicates that the fungus has successfully established itself within the biomass and begun producing hydrolytic enzymes. Figure 2F depicts rod-shaped structures on the surface of the hydrolyzed sugarcane bagasse. During the hydrolysis process, cellulose nanocrystals can be released, presented as rod-shape structures (Nang Vu et al., 2024). Overall, these observations show the degradation of lignocellulosic biomass throughout the fermentation period by *P. brevicompactum*.

The observed changes in the SCB biomass were anticipated given the high levels of enzyme production during solid-state fermentation (SSF). The target enzymes are key lignocellulosic degraders of both cellulose and hemicellulose fractions, which account for the degradation noted over the 7-day fermentation period. Mili et al. (2023) reported a similar pattern, in which a fungal consortium effectively hydrolyzed the SCB and altered its structure over a 29-day fermentation period. They detected signs of structural changes in the cell wall, including ruptured vascular bundles, fibers, and disrupted parallel stripes. Similarly, Scarcella et al. (2023) found that their crude enzymatic extract, rich in cellulases and hemicellulases, successfully degraded sugarcane bagasse, resulting in a modified structure.

FTIR spectroscopy was also used to examine the main structural transformations caused by the *P. brevicompactum* enzymatic actions in SCB after 7 days of fungal cultivation in solid-state fermentation. FTIR, a simple and rapid technique, is well known for qualitative and quantitative determination of lignocellulosic components. The FTIR spectra of both raw and fermented SCB is shown in Figure 3 and Table 3.

Figure 3: FTIR spectra of (a) SCB without fermentation (raw material); (b) SCB fermented by *P. brevicompactum*



Legend: Wavenumber (cm^{-1}) peak identification: 1730 – carboxylic groups from pectin, lignin, and hemicellulose; 1515 – aromatic C = C vibrations from lignin; 1457 – vibrations of CH3 and CH2 groups from lignin and carbohydrates; 1256 - C=O stretching of the guaiacyl ring from lignin; 1110 - vibration of the C-O-C glycosidic linkage in cellulose, as well as the plane deformation vibration of the aromatic C-H bond in lignin; 898 - C-O stretching vibrations and the vibrations associated with the β-glycosidic bonds in cellulose and hemicellulose.

Each spectrum displays characteristic bands that correspond to the composition of sugarcane bagasse, which includes lignin, hemicellulose, and cellulose.

The primary bands identified by FTIR in the samples include a broad band centered at 3430 cm^{-1}, indicative of the vibrations of hydroxyl groups, such as the O-H stretching of cellulose, O-H groups in lignin, and O-H bonds in pectin. The bands observed between 2900 and 2830 cm^{-1} are attributed to the asymmetric and symmetric vibrations of C-H groups. The band at 1730 cm^{-1} corresponds to the vibrations of carboxylic groups found in pectin, lignin, and hemicellulose. Additionally, the band at 1640 cm^{-1} is related to the stretching of the C=C bond in alkenes. The band at 1515 cm^{-1} pertains to the aromatic C=C vibrations of the benzene

ring characteristic of lignin, while the band at 1457 cm^{-1} is associated with the asymmetric deformation vibrations of $-CH_3$ and $-CH_2$ groups present in lignin and carbohydrates.

The spectral band observed at 1375 cm^{-1} is attributed to the overlapping contributions from C-H bending and C-O stretching vibrations within the aromatic rings of polysaccharide compounds, alongside aliphatic C-H stretching modes characteristic of cellulose, hemicellulose, and lignin. The bands identified at 1330 cm^{-1} and 1256 cm^{-1} correspond to the C-O stretching vibrations associated with the syringyl ring and the C=O stretching of the guaiacyl ring, respectively, which are components present in lignin. Furthermore, the spectral feature at 1160 cm^{-1} is linked to the asymmetric C-O-C stretching vibrations of the pyranose ring found in cellulose and hemicellulose. The band at 1110 cm^{-1} is attributable to the vibration of the C-O-C glycosidic linkage in cellulose, as well as the plane deformation vibration of the aromatic C-H bond in lignin. Additionally, the bands observed between 1060 and 1040 cm^{-1}, as well as the band at 898 cm^{-1}, are associated with C-O stretching vibrations and the vibrations associated with the β-glycosidic bonds in cellulose and hemicellulose.

A comparative analysis of the FTIR spectra reveals no significant alteration in the positions of the bands in the spectrum of fermented sugarcane bagasse when juxtaposed with that of in-natura sugarcane bagasse. This finding suggests that the molecular structure of the fermented sugarcane bagasse remains intact. However, a notable reduction in the intensity of all bands post-fermentation is observed when compared to the intensity observed in the in-natura sample. This decrease indicates that fermentation likely led to bond cleavage within the structural components of sugarcane bagasse, reflecting a degradation of cellulose, hemicellulose, and lignin. To further assess the extent of degradation instigated by fermentation, we conducted a thorough evaluation of the reduction in FTIR bands associated with the functional groups that denote cellulose, hemicellulose, and lignin in sugarcane bagasse. The results of the observed percentage decrease in the analyzed bands are presented in Table 3.

Table 3: Percentage reduction of specific SCB region peaks from control and fermented samples

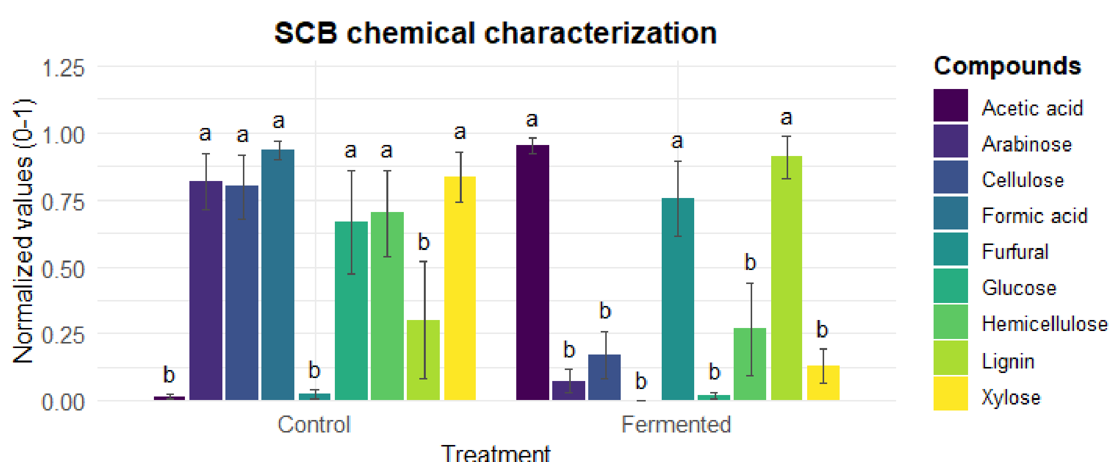
Wave lenght	**% reduction**	**SCB fraction**
1730	41.6	Hemicellulose degradation
1515	21.7	Lignin degradation
1457	40	Lignin degradation
1246	50	Lignin degradation
1110	57.2	Cellulose degradation
898	50.4	Cellulose degradation

The results shown in Table 3 indicate that, after fermentation, the intensity of the band at 1730 cm^{-1}, which is attributed to hemicellulose, decreased by approximately 42%. Similarly, the intensities of the bands at 1600 cm^{-1} and 1515 cm^{-1}, associated with lignin, decreased by 43% and 22%, respectively. Additionally, there was a notable change in the intensity of the bands corresponding to cellulose. Specifically, the intensity of the band at 898 cm^{-1}, linked to C-H deformation in cellulose, showed a decrease of 50% compared to the corresponding band in the in-natura sample. This variation likely reflects the differences between the outermost lignin layer and the inner layer, as well as the challenges in accessing the internal layer.

Namnuch et al. (2020) reported similar findings in their study, which investigated an *Aspergillus flavus* strain. They observed a 30% reduction in specific peaks in the FTIR spectra of SCB as a result of fermentation. Our results suggest that the enzymes produced during fermentation by *P. brevicompactum* played a significant role in the transformation of sugarcane bagasse.

To further support the theory that enzymes play a crucial role in the degradation of sugarcane bagasse, we characterized the sugars, organic acids, furfural, hydroxymethylfurfural, lignin, hemicellulose, and cellulose in SCB before and after fermentation. The results are shown in Figure 4 and Table 3.

Figure 4: SCB chemical characterization using normalized values for acetic acid, arabinose, cellulose, formic acid, furfural, glucose, hemicellulose, lignin, and xylose before and after fermentation (65% moisture and 7 days) at 23 °C and pH 5



Legend: The statistical analysis revealed significant differences in SCB composition between treatments (t-test, $p < 0.05$). Different letters above the bars indicate statistically distinct groups.

The chemical characterization analysis of SCB showed significant differences between the control and fermented treatments for all compounds evaluated. In the control treatment, acetic acid, cellulose, glucose, hemicellulose, and lignin were notably higher, while the

fermented treatment had higher levels of arabinose, formic acid, furfural, and xylose. These results suggest that fermentation significantly altered the SCB's chemical composition, decreasing certain structural carbohydrates while increasing others, affecting the lignocellulosic matrix. Statistical differences were determined through mean comparisons, through the t-test ($p < 0.05$) with distinct letters denoting statistically different groups.

Table 4: Real values and the standard deviation for each compound in the control and fermented treatments

Compound	Control	Fermented
Glucose	0.917 ± 0.210	0.508 ± 0.014
Xylose	0.450 ± 0.054	0.215 ± 0.037
Arabinose	0.024 ± 0.004	0.009 ± 0.002
Furfural	0.000 ± 0.000	0.001 ± 0.000
Acetic acid	0.643 ± 0.126	8.696 ± 0.411
Formic acid	0.020 ± 0.001	0.000 ± 0.000
Lignin	20.719 ± 1.968	23.875 ± 0.691
Cellulose	62.867 ± 2.203	56.221 ± 1.645
Hemicellulose	23.183 ± 1.255	21.223 ± 1.348

SCB fermentation using *P. brevicompactum* resulted in notable biochemical and structural changes in the substrate. The significant reduction in glucose, xylose, and arabinose levels demonstrates the strain’s ability to metabolize both hexose and pentose sugars. This sugar consumption is consistent with the known metabolic versatility of *P. brevicompactum*, which utilizes a broad spectrum of carbohydrates to support growth and secondary metabolite production (Marotti et al., 2017).

Additionally, the concentration of acetic acid significantly increased after fermentation. This is probably because acetic acid was one of the primary byproducts formed during the process, which is typical in fermentations involving lignocellulosic biomass, such as SCB (Rasmussen et al., 2014). Conversely, formic acid, which was present in the control sample, was undetectable after fermentation, implying that it was either consumed or converted into other compounds. Furfural, absent prior to fermentation, appeared in trace amounts afterward, likely resulting from the lignocellulosic biomass degradation at mildly acidic environments, albeit at very low levels (Rasmussen et al., 2014).

Regarding the structural components of the bagasse, a decrease in cellulose and hemicellulose contents was observed after fermentation, indicating that a portion of these polysaccharides was degraded during the process (Silvério, 2013). In contrast, lignin exhibited a relative increase in concentration. This is attributed to its recalcitrant nature; as cellulose and

hemicellulose are degraded, lignin becomes proportionally more abundant in the residual biomass (Cesarino et al., 2012).

4. Conclusions

Overall, the *P. brevicompactum* strain proved to be an efficient producer of cell wall-degrading enzymes-including pectinase, carboxymethylcellulase, total cellulase, and xylanase-when cultivated on SCB under optimized solid-state fermentation conditions. The maximum enzyme activities for all the tested enzymes were obtained at 65% moisture and 7 days of fermentation.

Besides enzyme production, fermentation resulted in biochemical and structural changes in the substrate: there was a reduction in fermentable sugars (glucose, xylose, arabinose), an increase in acetic acid, and the disappearance of formic acid, indicating active metabolic conversion of the lignocellulosic biomass by the fungus. Furfural was detected in trace amounts post-fermentation, likely as a byproduct of hemicellulose degradation. Structural analyses revealed substantial degradation of cellulose and hemicellulose, while lignin content increased proportionally, reflecting its recalcitrant nature.

This study shows that using *P. brevicompactum* might be a promising and cost-effective alternative for the bioconversion of lignocellulosic waste into value-added enzymatic products. There’s still work to do, however, the findings support the use of SCB as a sustainable substrate for industrial applications. Thus, future work should focus on further optimizing the production of β-glucosidase and avicelase, exploring the synergistic action of the enzyme cocktail, and scaling up the process for commercial implementation.

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ARTIGO 2 - P*enicillium brevicompactum* as a biocontrol agent: enzyme production, antifungal and nematicidal activities in solid-state fermentation

Redigido conforme norma do periódico científico - Versão preliminar	
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Periódico:	Enzyme and Microbial Technology (IF: 3.4)
ISSN	0141-0229

Abstract

As the global population grows, enhancing food production becomes urgent. A major challenge is pests, especially phytopathogenic fungi and nematodes that damage crops. While chemicals are commonly used, there’s a strong demand for sustainable alternatives, such as using fungi and bacteria as biological control agents. In this study, we evaluated the production of a crude extract fermented by *Penicillium brevicompactum*, using sugarcane bagasse as a substrate. After seven days of fermentation at 65% moisture, the extract contained approximately 257 U/mL of protease and 100 U/mL of chitinase. We assessed both antifungal activity and direct confrontation assays against five phytopathogenic strains and *Panagrellus* sp. larvae. Although the efficacy varies with different methods and microorganisms, both the fungus and its extract demonstrate promising antifungal activity (> 60%). Additionally, we observed nematicidal activity from both the crude enzymatic extract and the fungal strain against *Panagrellus* sp., achieving nearly a 75% reduction. Thus, *P. brevicompactum* shows potential as a nematophagous strain. Future studies should focus on field analyses to improve the implementation of effective and environmentally safe bioinputs.

Keywords*: Panagrellus* sp; phytopathogenic fungi; biocontrol; protease; chitinase

1. Introduction

Sugarcane bagasse (SCB) is a byproduct of the sugar industry that is generated in large quantities. It serves as a valuable raw material for manufacturing new products due to its low processing costs and high-quality outputs (Yadav et al., 2022). Given its abundance, SCB can be utilized in various applications, including biofuel, enzyme, paper, textile, and fertilizer production. Additionally, SCB is considered a sustainable alternative because it is biodegradable, recyclable, and reusable.

One specific application of SCB is enzyme production through solid-state fermentation, where microorganisms use SCB as the substrate and carbon source (Alokika et al., 2021). The primary enzymes produced during this fermentation process using lignocellulosic biomass include cellulases, hemicellulases, and ligninases. However, the type of fungi strain and the culture media used in fermentation can also result in the production of other enzymes (de Oliveira Rodrigues et al., 2020). The process of producing enzymes via solid-state fermentation is often cost-effective, as it utilizes residues like SCB. This process generates a crude enzymatic extract that is rich in enzymes and other metabolites, which can be applied for various purposes (Ajala et al., 2021).

A notable application is in the biological control of agricultural pests (Lv et al., 2024). Agricultural pests, such as phytopathogenic fungi and nematodes, pose significant challenges to agriculture, threatening various crops and ultimately impacting food safety and production (Amichot et al., 2024). *Penicillium* species are involved in biocontrol by producing proteases (EC 3.4) and chitinases (EC 3.2.1.14), which act as key enzymes in these processes (Nogueira et al., 2022; Xie et al., 2021). Additionally, they function as biocontrol agents in pest management (El-Morsy et al., 2023). Traditionally, chemical inputs have been used to manage these pests and prevent losses in agriculture and the economy. However, recent research has increasingly focused on natural approaches, utilizing natural extracts or microorganisms to control these pests (Fei et al., 2025; Sarboland et al., 2025).

These extracts frequently contain chitinases and proteases that can kill and inhibit the growth of fungi and the survival of nematodes (Hamrouni et al., 2024). Additionally, microorganisms possess mechanisms to suppress pest growth through the production of secondary metabolites and organic volatile compounds (Alamoudi, 2023). Depending on the cultivation conditions, they can also act as predators of pests (Shah & Pell, 2003). However, there is a notable lack of information regarding new fungal species that can be utilized for biological control and are capable of producing extracts for this purpose. Therefore, this study

aims to investigate: (i) the production of chitinase and protease from *Penicillium brevicompactum*; (ii) the biological activity of the crude fermented extract over five phytopathogenic strains and *Panagrellus* sp. larvae; (iii) the potential of *P. brevicompactum* as a nematophagous fungi; and (iv) the direct confrontation of *P. brevicompactum* against five phytopathogenic strains.

2. Materials and methods

2.1 Culture conditions and solid-state fermentation

The *Penicillium brevicompactum* strain was isolated from Amazonian soil and available from the Culture Collection of the Department of Food Science of the Federal University of Lavras (UFLA), Minas Gerais, Brazil.

Penicillium brevicompactum was cultured in Malt Extract Agar medium (MEA), with the following composition (g/L): glucose (15), malt extract (15), and agar (15) and incubated at 25 °C for seven days. The inoculum was prepared by adding 10 mL of 0,1% Tween 80 to the fungal colony and spread using a Drigalski loop. Conidia counting was performed in a Neubauer chamber and standardized to a final count of 1.5×10^8 conidia/g.

The SCB was provided by Cachaçaria Carinhosa, located in Perdões, Minas Gerais (21°4’4.058” S 44°59’29.036” W). The samples were dried in an oven for 72 h, until they reached a constant weight. Then, the SCB was ground into 1.0-mm particles, stored in impermeable polypropylene plastic bags and kept at room temperature.

Solid-state fermentation (SSF) was performed in 250-mL Erlenmeyer flasks containing 15 g of sterilized SCB at 23 °C. The enzyme production media consisted of the following composition (g/L): sodium nitrate (9), monopotassium phosphate (2.25), potassium chloride (0.75), magnesium sulfate (0.75), iron sulfate (0.015), and zinc sulfate (0.015), according to the methodology proposed by de Souza Castro et al. (2024). The media was buffered with citrate-phosphate (0,1 M) solution at pH 5.0 and inoculated with the conidia suspension (1.5×10^8 conidia/g). Homogenization was performed with a sterile glass rod.

To obtain the crude extract, 75 mL of distilled water (in a ratio of 1:5) were added to each Erlenmeyer flask. The flasks were kept under constant agitation at 120 rpm for 60 minutes in an ice bath. Then, the samples were filtered using voile fabric, centrifuged at 11,180 xg for 15 minutes, transferred to Falcon tubes and stored in a freezer at -22 °C until quantification of enzymatic activity (Soares et al., 2012).

2.2 Enzyme assays

2.2.1 Chitinase (EC 3.2.1.14)

Chitinase activity was quantified through the measure of reducing sugars using DNS (3,5-dinitrosalicylic acid) method (Miller, 1959). The assay was composed of 50 µL of enzyme, 450 µL of Tris-HCl (50 mM; pH 7.0) and 500 µL of 1% colloidal chitin previously prepared according to the method of Braga et al. (2013), incubated at 37 °C for one hour. The reaction was stopped by adding 1 mL of DNS reagent. The amount of reducing sugars released was determined by recording the absorbance at 540 nm. A control was performed by adding denatured enzyme to the reaction mixture. One unit of chitinase was defined as the amount of enzyme required to release 1 µg of N-acetylglucosamine per minute under the assay conditions.

2.2.2 Protease (EC 3.4)

The protease activity was measured according to Reimerdes et al. (1979). The reaction mixture consisted of using 100 µL crude enzymatic extract, 400 µL of Tris-HCl (pH 7.5; 200 mM) and 500 µL of 1% (w/v) casein. The mixture was incubated at 37 °C for 30 minutes and the reaction was stopped by adding 1 mL of 10% (w/v) trichloride acetic acid.

The mixture was then centrifuged at 10,000 xg for 10 minutes and the absorbance was measured at 280 nm. One unit of protease was defined as the amount of enzyme required to release 1 µg of tyrosine per minute under the assay conditions.

2.3 Nematicidal activity

2.3.1 Crude enzymatic extract

The free-living nematodes *Panagrellus* sp. were cultivated following the methodology described by (Leite Sufiate et al., 2017) at the Applied Biochemistry and Biotechnology Laboratory. The nematodes were kept in the dark at room temperature (25 °C) for 7 days in jars containing oats and distilled water. To assess the activity of the extract against *Panagrellus sp.* larvae, three experimental groups were established, each consisting of eight replicates. The

first group served as the negative control and received 50 µL of distilled water combined with 50 µL of a nematode suspension containing approximately 70 larvae. The second group, designated as the positive control, received 50 µL of heat-denatured extract-prepared by boiling the extract at 100 °C for one hour-along with 50 µL of the nematode suspension. The third group, representing the treatment condition, received 50 µL of the active extract mixed with 50 µL of the nematode suspension.

All mixtures were prepared in microtubes, resulting in a final volume of 100 µL per tube. The tubes were then incubated at 28 °C in complete darkness to prevent any light-induced effects on the nematodes or the extracts. Nematode viability and motility were monitored at 24, 48, and 72 hours post-incubation. After these periods, the total number of live larvae present in each tube was counted by optical microscopy (4×), according to the methodology described by Soares et al. (2013). Immobile larvae with non-intact cuticles were considered dead.

The percentage of nematode reduction was calculated according to Equation 1:

$$\% \, reduction = \frac{larvae \; recovered \; from \; control - larvae \; recovered \; from \; treatment}{larvae \; recovered \; from \; control} \times 100$$

2.3.2 Nematophagous fungi

Penicillium brevicompactum was reactivated in agar plates 2% (w/v) through the surface spreading method and incubated at 25 °C for 14 days. Then, 200 µL of approximately 1440 larvae of *Panagrellus* sp. were added to the center of the plate and incubated in the dark at 28 °C for 24, 48, and 72 hours.

After this period, all the larvae that had not been subjected to predation were recovered in hemolysis tubes with the aid of the Baermann apparatus and were quantified by optical microscopy (4x), according to the methodology described by (de Souza et al., 2023).

The percentage of nematode reduction was calculated according to Equation 1, previously mentioned in section 2.3.1.

2.4 Antifungal activity

2.4.1 Crude enzymatic extract

Five phytopathogenic fungal strains were selected for the study: *Fusarium oxysporum*, *Fusarium solani*, *Aspergillus westerdijkiae*, *Aspergillus ochraceus*, and *Aspergillus*

carbonarius. These strains were provided by the Phytopathology Lab at the Federal University of Lavras.

The strains were reactivated in Malt Extract Agar (MEA) medium for 7 days at 25 °C. To prepare the conidia suspension, 10 mL of 0.1% Tween 80 was added to the fungal colonies, and the mixture was spread using a Drigalski loop. Conidia counting was then performed using a Neubauer chamber, standardizing the final concentration to 10^6 conidia/mL.

For *F. solani* and *F. oxysporum*, instead of using a conidia suspension, we used one agar disc (Ø 8.8 mm). For the other strains, the microdrop technique was employed, where 10 µL of the conidia suspension was added to three equidistant points on the Petri dish.

All experiments were conducted in triplicates. The control consisted of the same experimental conditions but without the crude enzymatic extract. The inhibition percentage was calculated according to Equation 2:

$$\%\, inhibition\ = \left(\frac{control - treatment}{control}\right) \times 100$$

Where, control = control colony diameter in cm; treatment = colony diameter after growing in the crude enzymatic extract.

2.4.2 Direct confrontation

The experiments were performed in two parallel inoculations for *P. brevicompactum* against the five phytopathogenic fungus combinations. Plates containing the phytopathogenic fungi or the *P. brevicompactum* strain alone were used as controls. The experiment was conducted in triplicate.

The cultivation and the conidia suspension standardization procedure were the same as mentioned in the previous section (2.4.1). The *P. brevicompactum* strain against each of the five phytopathogenic strains were inoculated into a position 1 cm from the center of the plate (Tamandegani et al., 2020).

The plates were kept at 25 °C for 7 days. After this period, the visible area of the *P. brevicompactum* colony and the total area occupied by each phytopathogenic fungus were measured. Colony diameters were used as a measurement tool to evaluate the antagonistic capacity of each isolate against the different phytopathogenic strains, as proposed in Equation 2, previously mentioned in section 2.4.2.

2.5 Data analysis

The data obtained were analyzed using the R Statistical Software (v.2024.12.1; R Core Team, 2021). The results were submitted to analysis of variance (ANOVA), and those detected as significant by the F test were evaluated by the Tukey's test. A significance level of 5% ($\alpha = 0.05$) was adopted for all.

The efficiency of the treatments in reducing the number of nematodes compared to each experimental time was performed using Tukey's test at the significance level of 5%. The efficiency of the treatments in reducing the fungal colony growth was also performed using Tukey's test at a significance level of 5%.

3. Results and Discussion

3.1 Enzyme production

Penicillium brevicompactum was cultivated on SCB through solid-state fermentation, resulting in a crude extract that exhibited significant chitinase and protease activity. After 7 days of fermentation, 257.13 ± 33.19 U/mL of protease and 101.22 ± 9 U/mL of chitinase were produced. These results indicate that the extract is rich in both enzymes, which are essential for degrading the cell walls of phytopathogenic fungi and the cuticles of nematodes. This highlights the biotechnological potential of the extract as a bioproduct for biological control.

This is the first report regarding the production of protease and chitinase from *P. brevicompactum*. It is important to highlight the role of both enzymes in pest management. The primary components of fungal and nematode cell walls are chitin and proteins, making chitinases and proteases essential for degrading various cell wall parts (Fetterer & Rhoads, 1993). The production of these enzymes is crucial for developing new bio-inputs for agricultural applications.

Previous research has extensively documented the production of chitinases and proteases by *Penicillium* spp. through solid-state fermentation using media supplemented with chitin and milk or casein (Binod et al., 2005; Suresh et al., 2014). However, our study demonstrated enzyme activity without adding any specific substrates to the medium, with sugarcane bagasse (SCB) serving as the primary carbon source. This finding underscores the

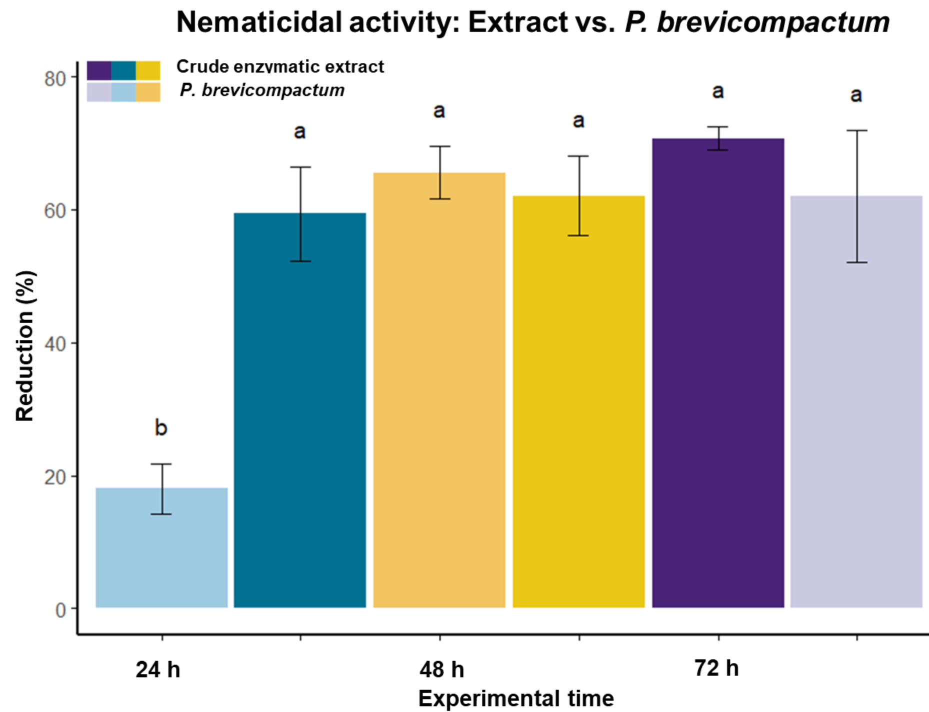
potential of *P. brevicompactum* to produce these enzymes, especially when culture conditions are optimized for enhanced protease and chitinase production.

3.2 Nematicidal activity of the crude enzymatic extract and *P. brevicompactum* as a nematophagous fungi

Both the crude enzymatic extract and the fungus *P. brevicompactum* demonstrated significant nematicidal activity against the larvae of *Panagrellus* sp. As shown in Figure 1, after 24, 48, and 72 hours of exposure to the active extract and the fungus, there was a substantial reduction in the number of viable nematodes.

Using the extract, 18% of the nematodes were eliminated after 24 hours. This increased to 65% after 48 hours and 70% after 72 hours. In the case of *P. brevicompactum*, 59% of the nematodes were destroyed after 24 hours, with 62% eliminated after both 48 and 72 hours. No significant differences were observed between the 48 and 72-hour incubation periods ($p < 0.05$) for both treatments (the extract and *P. brevicompactum*). The dead nematodes exhibited damaged cuticles, suggesting that the enzymes present in the extract had a direct impact on them (Figure 2).

Figure 1: Percentage reduction of *Panagrellus* sp. using both crude enzymatic extract and *P. brevicompactum* after 24, 48, and 72 hours

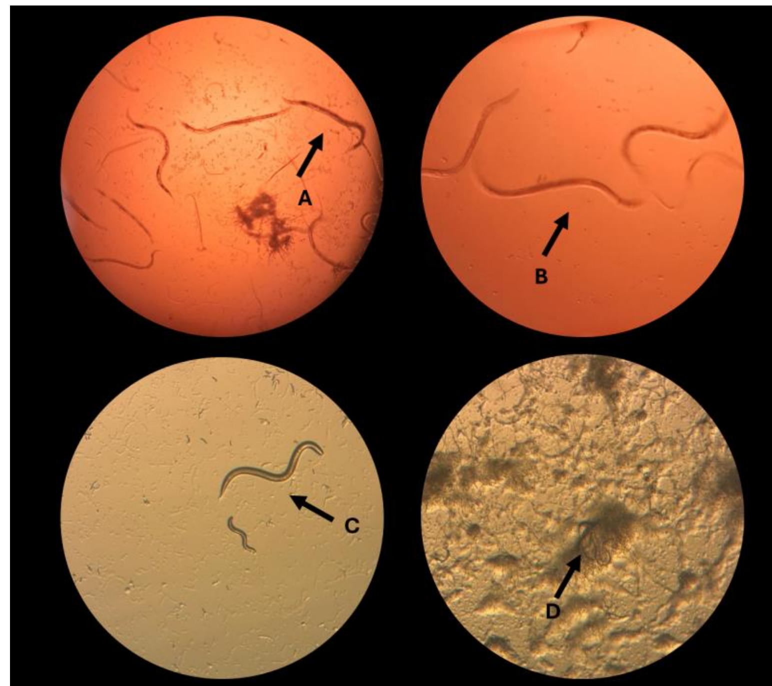


Legend: The statistical analysis revealed significant differences in % reduction across treatments and time (Tukey's test, $p < 0.05$). Different letters above the bars indicate statistically distinct groups.

Due to the presence of proteases and chitinases in the extract, it was anticipated that there would the destruction of *Panagrellus* sp. Studies indicate that enzymatic extracts, primarily composed of proteases, can destroy these nematodes by up to 76% to 80% (Castro et al., 2023b; A. C. Silva et al., 2025). The effectiveness of a crude enzymatic extract, or the fungi as predators, in the biological control of agricultural pests such as nematodes can be attributed to their ability to penetrate the cuticle through both mechanical pressure and enzymatic degradation of the key components of the cuticle, namely proteins and chitin (Fetterer & Rhoads, 1993).

Figure 2 shows the interactions between the fungus and the nematode, the crude enzymatic extract and the nematode, as well as the cuticle affected by their actions.

Figure 2: The larvae of *Panagrellus* sp. affected by the crude enzymatic extract containing chitinase and protease, as well as the influence of the fungus *P. brevicompactum*



Legend: A – indicates a destructed nematode resulting from enzymatic action and degraded cuticle; B – indicates the intact nematode in the control treatment; C – indicates the intact nematode in the control treatment within 2% agar; D – indicates the immobilized or predated nematode affected by *P. brevicompactum* hyphae in 2% agar.

After obtaining the percentage of intact larvae of *Panagrellus* sp. recovered with Baermann filtration, the treatments showed statistical significance ($p < 0.05$) for the experimental time of 24 hours. *Panagrellus* sp. is a free-living nematode frequently used in research due to its ability to thrive in diverse environments and its rapid life cycle, consisting of four developmental stages (Shokoohi & Shokoohi, 2024). They are non-parasitic and safe for laboratory use, making them suitable for research. Simple and economical to cultivate in oatmeal and water, *Panagrellus* sp. is often used in studies involving nematophagous fungi and extracts to assess predatory or toxic activities (A. T. da Silva et al., 2024). This model organism's ease of standardization enables reproducible experiments, providing valuable insights for biological control methods in agriculture.

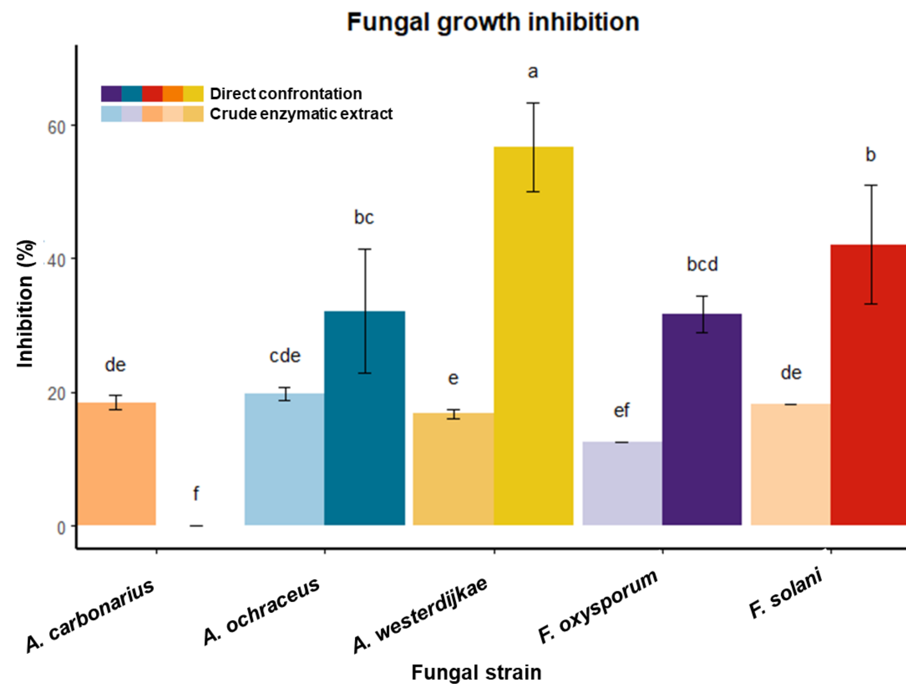
Our results show that *P. brevicompactum* might be considered a nematophagous species. To the best of our knowledge, this is the first report regarding this species being a nematophagous fungi. Sikandar et al., (2020) reported a *Penicillium* strain as potentially nematophagous against *Meloidogyne incognita*. Studying and discovering new species of nematophagous fungi is important for several reasons. Nematophagous fungi offer a sustainable alternative to chemical controls for nematodes. They use mechanisms like trap formation, where

hyphae create structures to capture nematodes (Figure 2), and direct parasitism, in which spores attach to or are ingested by nematodes (Y. Li et al., 2024). The fungus then penetrates the nematode's cuticle, consuming it until destruction. In this sense, discovering new nematophagous fungi improves biological control, promotes sustainable practices, and expands our understanding of these organisms.

3.3 Direct confrontation and antifungal activity of the crude enzymatic extract

The crude enzymatic extract was tested against five types of phytopathogenic fungi: *F. oxysporum*, *F. solani*, *A. westerdijkiae*, *A. ochraceus*, and *A. carbonarius*. The tests revealed that the extract effectively inhibited the growth of these fungi compared to the control group, demonstrating its antifungal activity. Additionally, in the direct confrontation tests, *P. brevicompactum* exhibited antagonism towards the phytopathogens, achieving high growth inhibition percentages. This indicates its ability to compete with and suppress the growth of the tested fungi. Figures 3 and 4 show the growth inhibition and direct confrontation results.

Figure 3: Fungal growth inhibition using both crude enzymatic extract and direct confrontation assays over five phytopathogenic strains



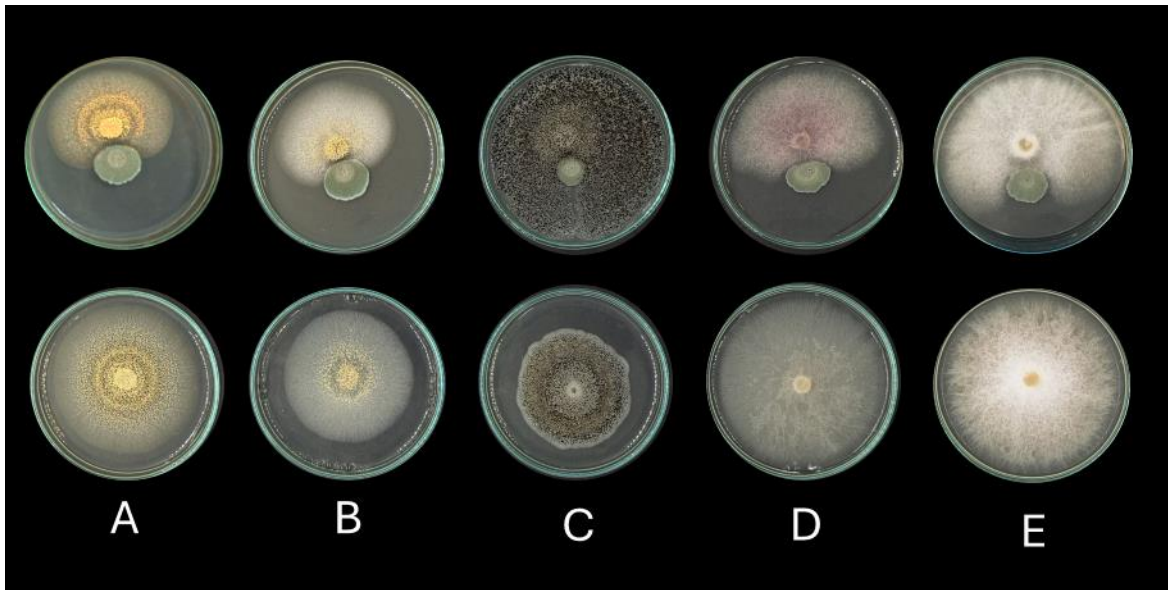
Legend: The statistical analysis revealed significant differences in fungal growth inhibition across treatments and strains (Tukey's test, $p < 0.05$). Different letters above the bars indicate statistically distinct groups.

The highest level of inhibition was observed for *A. westerdijkiae* during direct confrontation, indicating it was significantly more effective than all other treatments and strains. In contrast, *F. solani* exhibited significantly lower inhibition than *A. westerdijkiae* under the same treatment, although it still performed better than all the other groups. Intermediate levels of inhibition were found for *A. ochraceus* and *F. oxysporum* during direct confrontation, and *F. solani* during crude enzymatic extract.

A. ochraceus tested under enzymatic extract and *A. carbonarius* in direct confrontation showed smaller inhibition rates, which were not statistically different from each other, but both were lower than the more effective treatments. The lowest level of inhibition was recorded for *A. carbonarius* in the enzymatic extract, which was statistically lower than all other conditions, confirming it as the least effective combination.

Overall, the results indicated that direct confrontation consistently resulted in higher inhibition rates, particularly against *A. westerdijkiae* and *F. solani,* emphasizing that the efficacy of treatments varies significantly depending on the fungal strain. Figure 2 shows the relationship between *P. brevicompactum* as a biocontrol agent and each phytopathogenic strain.

Figure 4: Direct confrontation using *P. brevicompactum* against five phytopathogenic strains after 7 days of cultivation at 25 °C



Legend: A – *A. westerdijkiae* (control) and *A. westerdijkiae* against *P. brevicompactum*; B – *A. ochraceus* (control) and *A. ochraceus* against *P. brevicompactum*; C – *A. carbonarius* (control) and *A. carbonarius* against *P. brevicompactum*; D – *F. solani* (control) and *F. solani* agains *P. brevicompactum;* E – *F. oxysporum* (control) and *F. oxysporum* against *P. brevicompactum.*

Interestingly, *A. carbonarius* had a negative impact in the presence of *P. brevicompactum*; rather than inhibiting the strains, it actually promoted its growth. It is noteworthy that the crude enzymatic extract produced different effects on the tested strains compared to the direct confrontation assay, where the active fungal strain was utilized.

The potential of a strain as an antagonist is evaluated through the direct confrontation test. The primary parameters assessed include competition for space and nutrients, the production of metabolites with antifungal properties, and parasitism (Szekeres et al., 2006). In both the crude enzymatic extract and direct confrontation assays, we observed significant antagonistic activities.

The direct confrontation assays revealed physical interactions and competition, whereas the crude enzymatic extract assay highlighted the influence of chitinases and proteases, as well as other metabolites on fungal antagonism. During direct confrontation, inhibition mechanisms can operate in several ways. One explanation for growth inhibition is competition for space and nutrients, where the biocontrol fungus grows faster than the pathogen, thereby impeding its growth (Brazhnikova et al., 2025; Gloer, 1995). Another mechanism involves direct antagonism, where antifungal metabolites, such as enzymes, are produced to physically attack the pathogen. In our experiment with the crude enzymatic extract, we found that the extract contained metabolites capable of inhibiting pathogen growth (Thambugala et al., 2020). Thus,

multiple approaches can be employed to gain insights into the modes of action of fungal biocontrol agents (Tamandegani et al., 2020).

4 Conclusions

The present study demonstrated that *P. brevicompactum,* cultivated in sugarcane bagasse through solid-state fermentation, can produce a crude enzymatic extract rich in protease and chitinase. The extract achieved protease activities of approximately 257 U/mL and chitinase activities of 101 U/mL. These enzymes play a crucial role in degrading the cell walls of phytopathogenic fungi and the cuticle of nematodes, thus giving the extract significant biotechnological potential as a biocontrol agent.

Antifungal activity tests showed that both the enzymatic extract and *P. brevicompactum* exhibited the ability to inhibit the growth of various phytopathogenic fungi, with some inhibition rates exceeding 60%. Direct confrontation assays highlighted the competitive ability of the fungus, particularly against *A. westerdijkiae* and *F. solani*, although the effectiveness varied depending on the pathogen tested.

Additionally, both the extract and the fungus displayed significant nematicidal activity against *Panagrellus* sp., reducing the larvae number by up to 75 and 62% after 72 hours of exposure, respectively. The enzymatic action, evidenced by damage to the nematode cuticle, reinforces the role of proteases and chitinases in this process.

In summary, the findings confirm that *P. brevicompactum* can serve as a source of enzymatic extracts with antifungal and nematicidal properties. It also shows promise as a potential nematophagous fungus for use in biocontrol strategies. Further studies are necessary under field conditions to validate the efficacy and environmental safety of this bioproduct, aiming for its practical application in sustainable agriculture.

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ARTIGO 3 – Bioremediation of emerging herbicides by *Penicillium brevicompactum* enzymes

Redigido conforme norma do periódico científico - Versão preliminar	
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Periódico:	Bioresource Technology (I.F: 9.7)
ISSN	0960-8524

Abstract

The presence of atrazine and desethyl-atrazine in natural environments such as soil, air, and water poses a significant threat to human health and biodiversity. To explore a cost-effective strategy for pollutant degradation, we optimized the production of ligninases by varying moisture content and fermentation time. The optimal enzyme production was achieved at 65% moisture content after 7 days of fermentation. Following filtration and centrifugation, we obtained a crude enzymatic extract, which underwent dialysis for partial purification. We assessed the stability of both the crude and purified extracts over time, including their exposure to acetonitrile. A spiking solution of atrazine and desethyl-atrazine was prepared in acetonitrile at an initial concentration of 1 mg/L for degradation analysis. The experiment was conducted in 50-mL plastic conical tubes at 20 °C for 30 days, employing three treatments to facilitate pollutant degradation: partially purified enzymatic extract, crude enzymatic extract, and a crude extract devoid of enzymes. A crude enzymatic extract without the pollutants served as a negative control. Samples were collected over time to measure enzyme activity and the removal efficiency of each pollutant, along with their DT_{50}. Remarkably, nearly 75% of each pollutant was degraded after 21 days, underscoring the potential of the enzymatic extracts. Our findings suggest that enzymatic extracts may represent a more feasible and cost-effective approach for environmental pollutant degradation compared to traditional methods. These insights could inform future efforts to implement enzyme-based pollutant degradation in practical applications.

Keywords: atrazine; desethylatrazine; manganese peroxidase; laccase; lignin peroxidase.

1. Introduction

The growing demand driven by urbanization and human activity has spurred significant advancements in agriculture, medicine, and industry (Bisht et al., 2020). However, these developments are closely associated with the rise of emerging organic pollutants in the environment (Mishra et al., 2023). These pollutants, commonly detected in wastewater, groundwater, soil, and air, include a variety of chemical compounds such as pharmaceuticals, personal care products (PCPs), pesticides, and industrial additives chemicals (Jyoti Kalita et al., 2024).

Atrazine is a widely used s-triazine herbicide effective against broadleaf and grassy weeds in agriculture. It primarily degrades into desethyl-atrazine (DEA) and desisopropyl-atrazine (DIA) through various water treatment processes. While it effectively controls weeds, atrazine poses significant health and environmental risks. It is an endocrine disruptor linked to hormonal imbalances, reproductive issues, and cancers such as breast and prostate cancer (S. Singh et al., 2017). Its high solubility can contaminate water sources, affecting drinking water and aquatic life. Though banned in the EU since 2003, it remains in use in countries like Brazil, raising concerns for agricultural safety and public health (Arthur White, 2016).

Pesticides like atrazine can be remediated using various strategies, with enzymes playing a key role. Bioremediation employs microorganisms such as bacteria and fungi to degrade pesticides into less harmful substances through specific enzymes coded by specific genes that will degrade atrazine into CO_2 and ammonia (Mousavi et al., 2021a). Enzymatic bioremediation involves using isolated, cell-free enzymes that are mainly produced and extracted from microorganisms, allowing them to be applied directly to contaminated environments without needing specific conditions (Hu et al., 2024).

A good example is the ligninolytic enzymes, including peroxidases (EC 1.11.1.14; EC 1.11.1.15) and laccases (EC 10.1.3.2), are effective for degrading diverse organic pollutants and can be optimized by adjusting temperature, pH, and cofactors. These enzymes can also be immobilized for enhanced stability and reuse, making the process more efficient (Xia & Lin, 2022). However, there's limited information on their stability and activity in the presence of pollutants. This study aims to investigate: (i) optimal conditions for producing lignin peroxidase, laccase, and manganese peroxidase; (ii) the DT_{50} of atrazine and desethyl-atrazine in the enzymatic extract; (iii) first-order kinetics; and (iv) monitoring enzyme activity with atrazine and desethy-latrazine during the DT_{50} experiment.

2. Materials and methods

2.1 Culture conditions and substrate preparation

The *Penicillium brevicompactum* strain was isolated from Amazonian soil and available from the Culture Collection of the Department of Food Science of the Federal University of Lavras (UFLA), Minas Gerais, Brazil.

Penicillium brevicompactum was cultured in Malt Extract Agar medium (MEA), with the following composition (g/L): glucose (15), malt extract (15), and agar (15) and incubated at 25 °C for seven days. The inoculum was prepared by adding 10 mL of 0,1% Tween 80 to the fungal colony and spread using a Drigalski loop. Spore counting was performed in a Neubauer chamber and standardized to a final count of 1.5×10^8 spores/g.

The SCB was provided by Cachaçaria Carinhosa, located in Perdões, Minas Gerais (21°4'4.058" S 44°59'29.036" W). The samples were dried in an oven for 72 h, until they reached a constant weight. Then, the SCB was ground into 1.0-mm particles (18 mesh), stored in impermeable polypropylene plastic bags and kept at room temperature.

2.1.1 Optimization of enzyme production through a rotational central composite design (CCRD)

The optimization of cell-wall degrading enzyme production was carried out using the central composite rotational design (CCRD) experimental framework, focusing on time (days) and moisture percentage (%) as independent variables. These factors were chosen for their significance in solid-state fermentation and enzyme production (A. T. da Silva et al., 2025). The impact of these variables on the response variable, which is the production of cell-wall degrading enzymes, was analyzed at five experimental levels: time intervals of 24, 21, 14 (the central point), 7, and 4 days, and moisture levels of 63, 65, 70 (the central point), 75, and 77%. A total of thirteen experiments were conducted, including five replications at the central point, as detailed in Table 1.

Table 1: Experimental design matrix, considering two variables (time and moisture) and five repetitions at the central point, totaling 13 experiments

Run	Time (days)	Moisture (%)
1	7	65
2	21	65
3	7	75
4	21	75
5	4	70
6	24	70
7	14	63
8	14	77
9	14	70
10	14	70
11	14	70
12	14	70
13	14	70

Solid-state fermentation (SSF) was performed in 250-mL Erlenmeyer flasks containing 15 g of sterilized SCB at 23 °C. The enzyme production media consisted of the following composition (g/L): sodium nitrate (9), monopotassium phosphate (2.25), potassium chloride (0.75), magnesium sulfate (0.75), iron sulfate (0.015), and zinc sulfate (0.015) (de Souza Castro et al., 2024d). The media was buffered with citrate-phosphate (0,1 M) solution at pH 5.0 and inoculated with the spore suspension (1.5×10^8 spores/g). Homogenization was performed with a sterile glass rod.

To obtain the crude extract, 75 mL of distilled water (in a ratio of 1:5) were added to each Erlenmeyer flask. The flasks were kept under constant agitation at 120 rpm for 60 minutes in an ice bath. Then, the samples were filtered using voile fabric, centrifuged at 11,180 xg for 15 minutes, transferred to Falcon tubes and stored in a freezer at -22 °C until quantification of enzymatic activity (Soares et al., 2012)

To obtain the dialyzed extracts, we utilized a portion of the prior crude extract and performed dialysis against distilled water for 72 hours at 4 °C, ensuring constant agitation. This was carried out using dialysis tubing (33 mm x 21 mm), following the method established by Llerena-Suster et al. (2014), with modifications. This procedure was implemented to reduce the concentration of metabolites and peptides in the extract.

2.2 Enzyme assays

Laccase (EC 10.1.3.2) was assayed according to (Hou et al., 2014), with modifications: Citrate phosphate buffer pH 3.0, 100 mM: 100 μL; ABTS (2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]; $\varepsilon = 36{,}000\ M^{-1}\cdot cm^{-1}$), 2mM: 100 μL; Enzyme extract: 100 μL). Samples were read at 420 nm, at 0 and 15 minutes in the dark. Enzyme activity was calculated in U/L according to Equation 1, which defines a unit of enzymatic activity (U) as 1 mM of oxidized ABTS per minute.

Lignin peroxidase (LiP: EC 1.11.1.14) was assayed according to (Vrsanska et al., 2016), with modifications: Sodium tartrate buffer, pH 3.0, 125 mM: 260 μL; Veratryl alcohol ($\varepsilon = 9300\ M^{-1}\cdot cm^{-1}$), 25 mM: 300 μL; H_2O_2 50 μM: 10 μL; Enzyme extract: 10 μL). Samples were read at 310 nm immediately after the addition of the enzyme. The enzyme activity was determined in U/L according to Equation 1, which defines a unit of enzymatic activity (U) as 1 mM of oxidized veratryl alcohol per minute.

Manganese peroxidase (MnPase: EC 1.11.1.15) was assayed according to (Kuwahara et al., 1984),with modifications: Phenol red ($\varepsilon = 22{,}000\ M^{-1}\cdot cm^{-1}$), 0.1%: 10 μL; Sodium Lactate, 0.25 M: 10 μL; MnSO4, 2 mM: 50 μL; Bovine Serum Albumin, 0.5%: 20 μL; H_2O_2, 2 mM: 20 μL; Enzyme extract: 100 μL; NaOH, 1 M: 50 μL to stop reaction). Samples were read at 610 nm after 4 minutes. The difference between the absorbance from the control (no enzyme) and the crude enzymatic extract samples was calculated, and the enzyme activity was determined in U/L according to Equation 1, which defines a unit of enzymatic activity (U) as 1 mM of oxidized phenol red per minute.

$$\text{enzyme activity} = 10^6 \frac{A_\lambda * V}{v * t * \varepsilon} \qquad (1)$$

Where, A_λ= absorbance at a specific wavelength λ (AU); V = total reaction volume (L); v = enzymatic extract volume (L), t = time of reaction; ε = molar extinction coefficient ($M^{-1}.cm^{-1}$).

2.3 Atrazine and desethylatrazine DT_{50} set-up

Based on their environmental significance, atrazine and desethylatrazine were selected as organic pollutants for this study.

The degradation experiment was conducted in sterile 50 mL plastic conical tubes. Fifteen mL of the crude and dialyzed enzyme extract was added to each flask, followed by the OMP spiking solution (acetonitrile) to reach a final 1 mg/L concentration. In treatments without

the pollutants, the spiking solution was replaced by acetonitrile and sterile distilled water, occurring as a control to monitor the enzymatic stability.

All bottles were incubated at 20 °C and 150 rpm for 30 days. The experiment was conducted in triplicate, and samples were collected after 0, 1, 3, 9, 15, 21, and 27 days and stored in Eppendorf tubes in the dark at –22 °C freezer until quantification.

2.4 Sample preparation and atrazine and desethyl-atrazine quantification

Samples were prepared using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction process. First, 500 µL of the enriched sample (7.5 mg/L) was mixed with 2 mL of H_2O, 1 mL of acetonitrile, 0.25 g of NaCl, and 0.5 g of $MnSO_4$, and then the mixture was homogenized in a vortex for 30 seconds. Next, it was centrifuged at 4400 rpm for 15 minutes, and the upper phase was collected and transferred to Eppendorf tubes, where it was lyophilized (do Amaral et al., 2022). Finally, the lyophilized samples were reconstituted with 500 µL of acetonitrile before being injected into the liquid chromatograph.

The pesticide determination in the enzymatic extract was assessed by liquid chromatography equipped with a diode-array detector (DAD 3000). Chromatographic separations were conducted in a reversed-phase column Gemini C18 (250 × 4.6 mm – 5 µm) with a guard column of the same phase. Acetonitrile (A) and water (B) were used in gradient elution mode as the mobile phase: 20% of A reaching 90% in 15 min, remaining constant in this condition for 15 min. In the column cleaning step, the mobile phase returned to 20% of A in 2 min, remaining under this condition for 5 min until a new chromatographic run was initiated. The flow rate was 0.4 mL min^{-1}, the column temperature was set to 25.0 ± 1.0 °C, and the injection volume was 20 µL. The following wavelengths (λ) were monitored to detect the pesticides: 220 nm was used to ATZ and DEA, identified through the retention time and comparison of the UV spectra of the analytes with known standards (Gabardo et al., 2021).

2.5 Data analysis

The optimization matrix, together with all analyses—including response surface, ANOVA, F-test, and contour plots—was generated using R Statistical Software (v.2024.12.1; R Core Team, 2021). Enzyme activity monitoring and removal efficiency were analyzed using the Tukey test ($p < 0.05$) and visualized with R. The DT_{50} values were calculated based on Equation 2.

$$DT50 = \frac{\ln(2)}{k} \quad (2)$$

Where, DT50 = half-life time (the time it takes for the concentration of the substance to be reduced to half the initial value); ln(2) = the natural logarithm of 2; k = rate constant, which indicates the speed at which the reaction occurs. The T test was applied to observe the significance ($p < 0.05$) of the treatments.

3. Results and discussion

3.1 Enzyme optimization

Thirteen experiments were conducted using a central composite rotational design (CCRD) to evaluate the impact of fermentation time (4, 7, 14, 21, and 24 days) and moisture content (63%, 65%, 70%, 75%, and 77%) of sugarcane bagasse (SCB) as a substrate for the enzyme production by *P. brevicompactum*. The objective was to identify the optimal conditions for the simultaneous production of ligninolytic enzymes: laccase, lignin peroxidase (LiP), and manganese peroxidase (MnPase). After excluding terms related to non-significant variables ($p < 0.05$), the equation for the final response (enzyme activity) is presented in Table 2. The F test confirmed the significance of the regression model statistics, and analysis of variance (ANOVA) was employed for the quadratic response surface model. The regression coefficients underscored the significance of the model, yielding an R^2 value of > 0.87 for the significant variables.

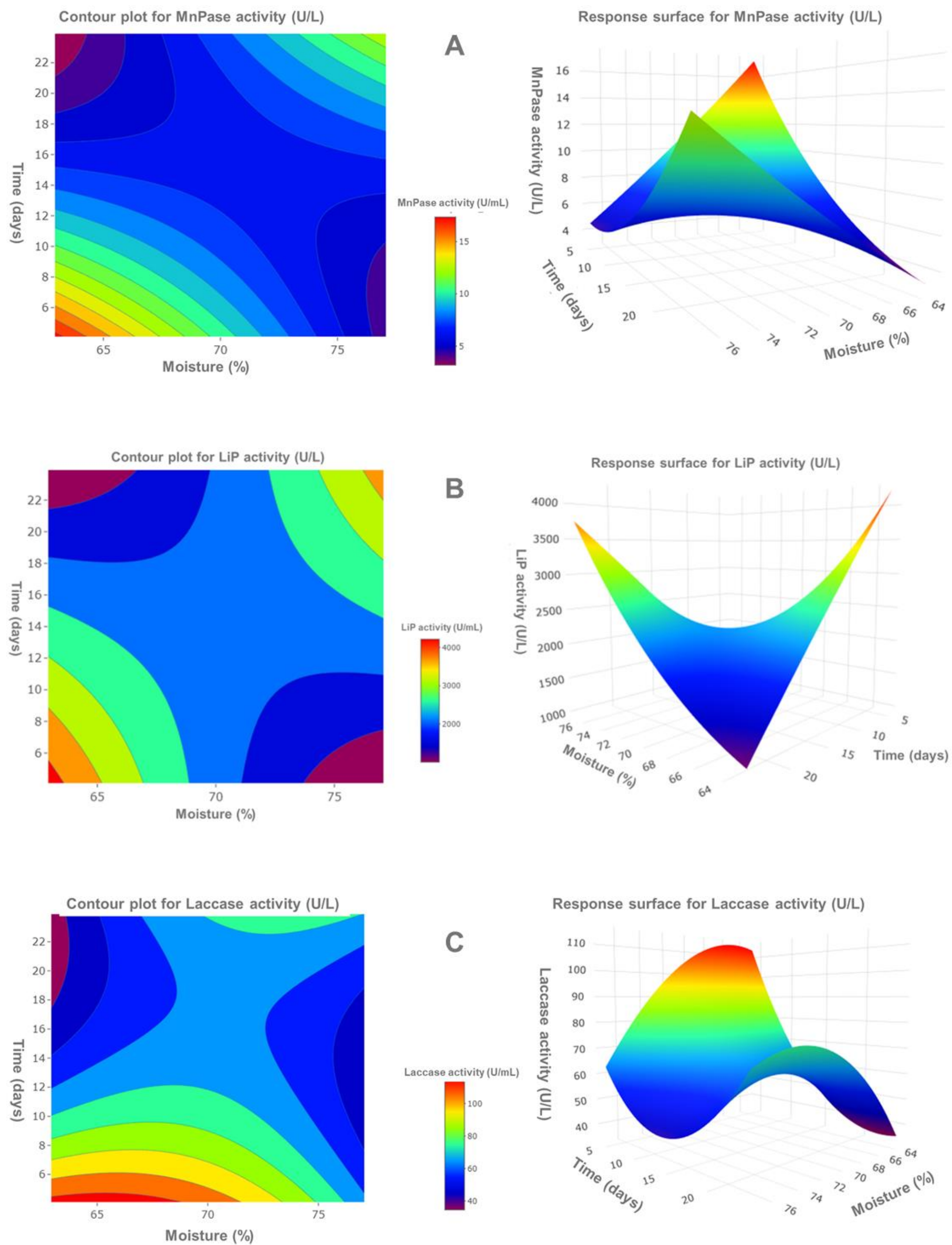
Response surface plots for all the enzymes were constructed despite their significant interactions that showed a positive effect on enzyme production. They are presented in Figure 1. Three dimensional graphs (Fig. 1A, B, C) showed a significant effect on interactions between these parameters on ligninases production. The response surfaces generated for each enzyme showed that both fermentation time and moisture content acted synergistically, leading to increased activities of laccase, LiP, and MnP. The surface and contour plots confirmed that maximum production of these three enzymes occurs under similar conditions, supporting the hypothesis that it is possible to optimize their joint production in a single fermentation process.

The optimal conditions identified were a moisture content of 65% and a fermentation time of 7 days. Under these conditions, the highest activity levels for laccase, LiP, and MnP were achieved, as detailed in Table 2.

The model was validated, and three pre-established experimental conditions from the matrix were repeated: 14 days at 70% moisture content, 4 days at 70% moisture, and 7 days at

65% moisture. The results were consistent with our observations from the experimental conditions, showing variations of approximately 5% for all the tested enzymes.

Figure 1: Response surface and contour plots for the activity of all significant models: MnPase, LiP, and Laccase, under two different variables: time and moisture content



Legend: A – response surface and contour plots for MnPase; B response surface and contour plots for LiP; C – response surface and contour plots for Laccase.

Table 2: Final response equations from the surface response design and each enzyme's R^2 and activity (U/mL) at the optimal conditions (65% (w/w) moisture content and 7 days of fermentation)

Enzymes	Equation	R2	Activity (U/L)
Laccase	$Y = 5018,002 + 0,080303X_2 + 0,019187X_1X_2$	0,96*	103.7
Lignin peroxidase	$Y = 60838,26 - 1361,7X_2 + 20,96768X_1X_2$	0.97	4000
Manganese peroxidase	$Y = 133,4967 - 2,18965X_2 + 0,080087X_1X_2$	0.87	13.64

Legend: Y – enzyme activity; X_1 – coded level for time; X_2 – coded level for moisture content; *Cubic model.

The production of laccases from *P. brevicompactum* has been reported by (de Souza Castro et al., 2024d). The production of lignin peroxidase and manganese peroxidase by other *Penicillium* species is well recognized (Govarthanan et al., 2017; Wulandari et al., 2013). Overall, all the ligninases such as lignin peroxidase and manganese peroxidase are widely produced by white-rot fungi and brown-rot fungi, majority of the basidiomycete group (Lundell et al., 2010; Ricaczeski et al., 2025).

Hariharan & Nambisan (2013) reported enzyme activities of 2,999 U/mL for lignin peroxidase (LiP), 896 U/mL for manganese peroxidase (MnPase), and 499 U/mL for laccase after 8 days of solid-state fermentation using pineapple leaves. They utilized the white-rot fungus *Ganoderma lucidum*, which likely accounts for the significantly higher enzyme activities compared to those observed in the current study.

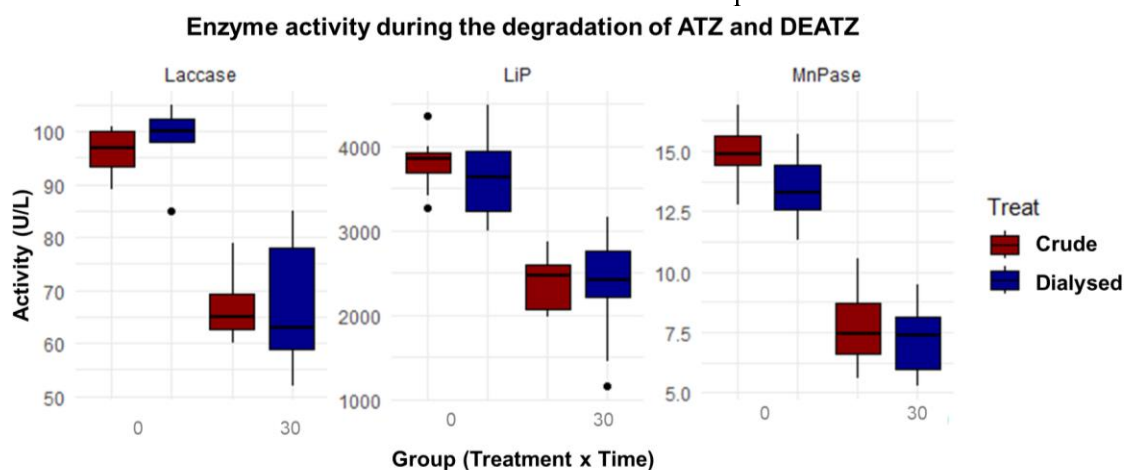
The success of the optimization model may be influenced by the selected variables analyzed during solid-state fermentation. These values are specific to each system, which consists of microorganisms, substrates, and desired metabolites. Low moisture content limits microbial growth and decreases substrate availability. Conversely, high moisture content reduces the medium's porosity and disrupts oxygen diffusion (Wang et al., 2023). Time is also a crucial parameter; extended fermentation can lead to nutrient depletion and enzyme adsorption. The less fastidious the microbial metabolism is, the shorter the fermentation time required (S. F. D. M. Santos et al., 2008).

3.2 Atrazine and desethyl-atrazine degradation

3.2.1 Enzyme activity monitoring

The boxplot presented in Figure 2 illustrates that across all evaluated enzymes (laccase, LiP, and MnPase), both in the crude (red) and dialyzed (blue) treatments, there is a significant decline in enzymatic activity from time 0 to 30 days.

Figure 2: Enzyme activity during the degradation of ATZ and DEATZ in times 0 and 30 days of incubation at 20 °C and 150 rpm



For laccase, the initial mean values are relatively high and comparable between treatments, with a notable decrease after 30 days, lacking any pronounced visual differences between the crude and dialyzed treatments.

A similar trend is observed with the LiP enzyme, which also starts with high activity at time 0 but experiences a marked reduction at time 30, exhibiting greater variability and the presence of some outlier values.

In the case of MnPase, values also decline from time 0 to 30 days in both treatments, showing slightly higher initial means in the crude treatment, yet without any significant differences between the groups. Overall, the data suggest that time is the primary factor influencing the reduction in enzymatic activity, while the type of treatment (crude or dialyzed) does not yield consistent variations in enzyme averages throughout the evaluated period.

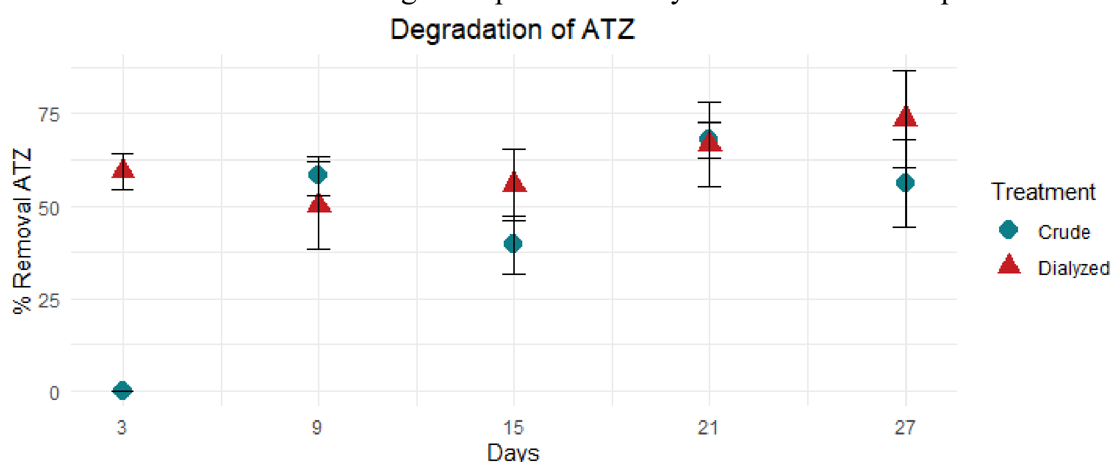
Time is a critical factor influencing enzyme stability, but the presence of organic solvents also plays an important role. The spiking process was evaluated using acetonitrile-based solutions that contained both atrazine and desethyl-atrazine. In this context, acetonitrile may have contributed to the partial denaturation of these extracts over time through a mechanism that destabilizes the non-covalent interactions responsible for maintaining their three-dimensional structure (Stepankova et al., 2013).

Although enzyme activity declined over the 30-day experiment, we were still able to observe high removal rates for both atrazine and desethyl-atrazine. Even at lower concentrations, enzymes likely play a crucial role in the degradation process (Kennes-Veiga et al., 2022b). To our knowledge, this is the first study investigating the degradation of atrazine and desethyl-atrazine using crude and dialyzed enzymatic extracts from *P. brevicompactum*.

3.2.2 Determination of the DT_{50}

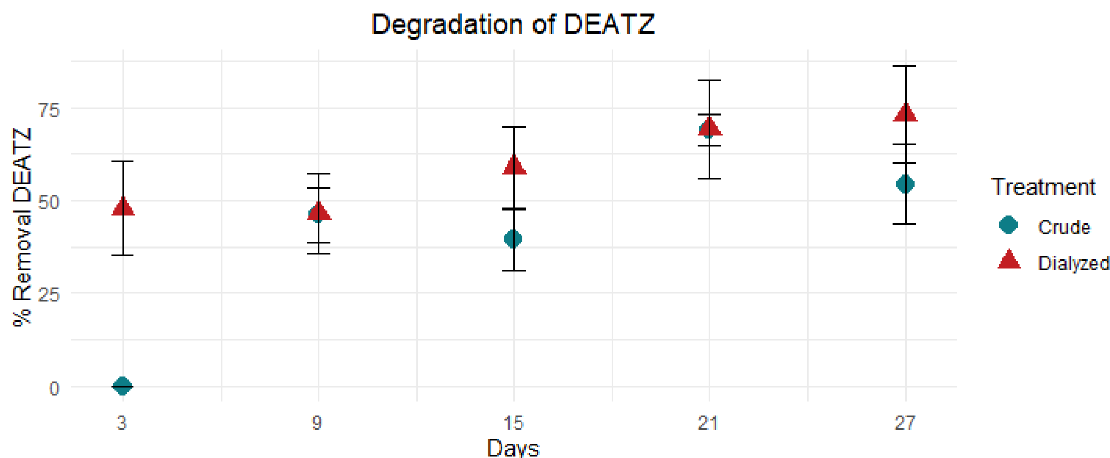
The crude extract, obtained under optimal conditions for enzyme production, was used to assess its capability to degrade atrazine and desethylatrazine. The quantification of the contaminants was performed using high-performance liquid chromatography (HPLC), which allowed for precise monitoring of the residual concentrations of atrazine and desethylatrazine over time. The results demonstrated that the crude and dialyzed enzymatic extracts effectively promoted significant degradation of both compounds during the incubation period, as shown in Figures 3 and 4.

Figure 3: Removal efficiency (%) of atrazine (ATZ) in both crude and dialyzed enzymatic extracts during 27 experimental days at 20 °C and 150 rpm



The degradation rate remained relatively linear throughout the 27 experimental days. By day three, we observed that at least 50% of the compound had degraded in the dialyzed enzymatic extract. In contrast, this same level of degradation was only observed on day nine for the crude enzymatic extract. Time was a significant factor, as the crude enzymatic extract showed significance ($p < 0.05$) on day 3, which was notably different from the other days.

Figure 4: Removal efficiency (%) of desethyl-atrazine (DEATZ) in both crude and dialyzed enzymatic extracts during 27 experimental days at 20 °C and 150 rpm

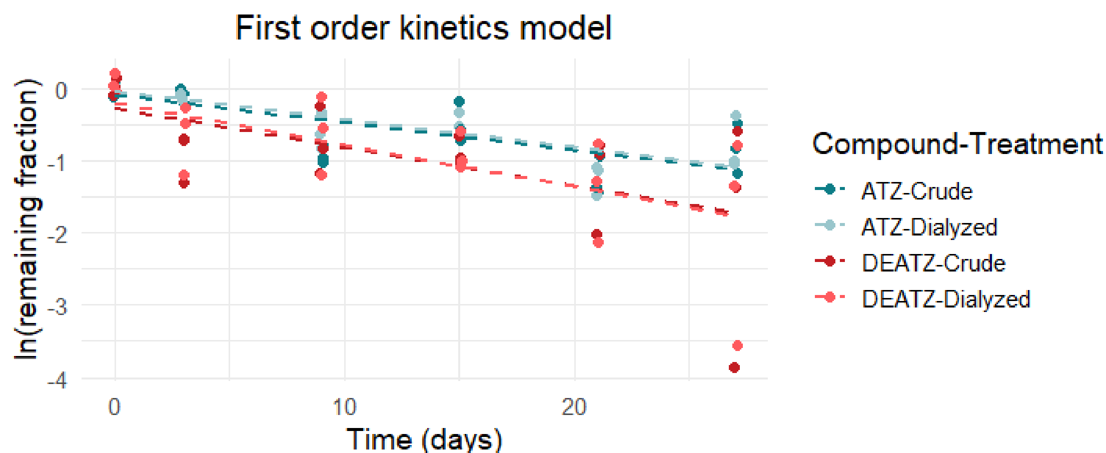


A similar trend was noted in the degradation of desethyl-atrazine. The degradation rate was relatively linear over the course of the 27 experimental days. Notably, by day 15, at least 50% of the compound had degraded in the dialyzed enzymatic extract. In contrast, the same level of degradation in the crude enzymatic extract was only observed by day 21. The same trend was observed for DEATZ, where time was a significant factor. The crude enzymatic extract showed significance ($p < 0.05$) on day 3.

These results underscore the significance of the removal efficiency of crude and dialyzed enzymatic extracts for atrazine and desethyl-atrazine. The persistence of these pollutants in the environment is closely related to their resistance to abiotic hydrolysis and their stability when exposed to sunlight. Additionally, they have limited potential for volatilization and are moderately susceptible to aerobic biodegradation (Rostami et al., 2021).

Analysis of the degradation data indicated that both contaminants followed first-order kinetics, meaning that the degradation rate was proportional to the residual concentration of the compounds, as shown in Figure 5.

Figure 5: First order kinetics model during enzymatic treatment of atrazine and desethyl-atrazine



From this data, it was possible to calculate the half-life (DT50) for both atrazine and desethyl-atrazine in the crude enzymatic extract, as shown in Table 3. The DT50 values obtained showed a considerable reduction ($p < 0.05$) in the time required for half of the initial concentration of the contaminants to degrade, underscoring the effectiveness of the crude extract produced by *P. brevicompactum*.

Additionally, monitoring of the enzymatic activity during the degradation experiment revealed that the enzymes maintained sufficient activity levels throughout the 30-day period, ensuring the ongoing degradation of the contaminants.

Table 3: Determination of DT50 (days) for each compound in each treatment during each sampling timepoint

Treatment	**Time**	**DT50 (days)**	
		Atrazine	**Desethylatrazine**
Crude	0	0 ± 0	0 ± 0
Dialyzed		0 ± 0	0 ± 0
Crude	3	0 ± 0	0 ± 0
Dialyzed		2.34 ± 0.5	3.73 ± 1.86
Crude	9	7.34 ± 1.99	10.74 ± 3.5
Dialyzed		10.75 ± 7.08	11.84 ± 7.39
Crude	15	23.23 ± 1.75	22.95 ± 9.13
Dialyzed		14.14 ± 7.43	13.23 ± 7.80
Crude	21	13.04 ± 3.07	12.56 ± 2.62
Dialyzed		14.23 ± 6.88	14.01 ± 9.49
Crude	27	26.55 ± 7.11	27.40 ± 6.37
Dialyzed		15.09 ± 9.82	15.37 ± 9.93

Understanding the half-life (DT50) of pollutants is essential for determining their persistence in various environments. For atrazine, the estimated half-life in the air is 14 hours,

and it can be removed through both humid and dry deposition. In soil, the half-life can range from 1.4 to 108 days due to microbial degradation, influenced by humidity and temperature (Agertved et al., 1992). In water, the half-life varies from 96 to 224 days, and the compound can even become recalcitrant (G. C. Li & Felbeck, 1972).

For desethyl-atrazine, the estimated half-life in the air is 21 hours, while in soil it is about 26 days, and in water, it exceeds 100 days (Krzyzanowski et al., 1996). The mechanisms of degradation for both atrazine and desethyl-atrazine are similar. Our findings indicate that both crude and dialyzed enzymatic extracts can serve as effective alternatives for treating environmental contaminants.

Recent studies have demonstrated the effectiveness of certain fungal strains in degrading atrazine and desethyl-atrazine through fermentation. For instance, the strain *Pleurotus ostreatus* was found to degrade atrazine by 82% and desethyl-atrazine by 71% (Lopes et al., 2020). In contrast, *Gloelophyllum striatum* only managed to degrade 37% of atrazine under similar fermentation conditions (Henn et al., 2020). While these findings highlight the potential of these fungal strains, our study emphasizes the effectiveness of a fermented extract that is rich in enzymes and proteins capable of effectively degrading both atrazine and desethyl-atrazine.

4. Conclusions

In this study, we demonstrated that the fungus *Penicillium brevicompactum* is capable of concurrently producing the enzymes lignin peroxidase, manganese peroxidase, and laccase through an optimized organic fermentation process. By employing a central composite rotational design (CCRD), we identified the optimal conditions for enzyme production: 65% moisture and a 7-day incubation period, during which all enzymes reached maximum activity, thereby confirming our hypotheses regarding the synergistic and simultaneous production of these biocatalysts. ANOVA analysis revealed that both incubation time and moisture content act synergistically to optimize enzyme production, with adjusted models exhibiting R^2 values greater than 0.87, indicating that the model was well-suited for the experiment.

The evaluation of the crude and partially purified enzymatic extract revealed that both maintain sufficient activity to promote the degradation of the pollutant’s atrazine and desethyl-atrazine, even with the gradual decrease in enzymatic activity over 30 days of incubation. The degradation experiment showed that approximately 75% of each contaminant was removed after 21 days, with removal rates higher than those observed under natural environmental conditions. The kinetic data indicated that the improvement of the first-order model, allowing

the calculation of the DT50 (half-life) of the compounds: both atrazine and desethylatrazine caused a significant reduction in the persistence time when treated with the enzymatic extract, compared to those reported for soil and water, where the half-life can exceed 100 days.

In addition, the use of agro-industrial waste as substrate and the obtaining of extracts rich in enzymes and metabolites reinforce the sustainable and low-cost nature of the approach. The enzymatic extract of *P. brevicompactum* was shown to be efficient not only in the production of multiple ligninases under optimized conditions, but also in the direct application for the removal of emerging organic contaminants, such as herbicides, in aquatic systems.

Therefore, our results demonstrate the biotechnological potential of the enzymatic extract of *P. brevicompactum* as a viable, efficient and sustainable solution for the bioremediation of persistent organic substances. The developed process can be adapted to different residues and contaminants, contributing to the implementation of innovative enzymatic technologies in the treatment of contaminated water and soil.

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TERCEIRA PARTE

3 CONSIDERAÇÕES FINAIS

Este estudo explorou o potencial biotecnológico de *Penicillium brevicompactum* cultivado em bagaço de cana-de-açúcar através da fermentação em estado sólido, com o objetivo de produzir extratos enzimáticos para uso em biocontrole, biorremediação e valorização de biomassa. O principal objetivo foi atender à crescente necessidade de métodos sustentáveis e econômicos para o manejo de resíduos agrícolas, o tratamento de poluentes ambientais e o desenvolvimento de agentes biológicos para combater fitopatógenos e nematoides.

O objetivo foi atingido, demonstrando que *P. brevicompactum* produziu com eficácia uma ampla gama de enzimas — incluindo protease, quitinase, pectinase, celulases, xilanase, lignina peroxidase, manganês peroxidase e lacase — em condições otimizadas de fermentação (65% de umidade, 7 dias de incubação). Isso corrobora a hipótese inicial sobre a capacidade do fungo de produzir simultaneamente diferentes enzimas, enquanto atua sinergicamente em diferentes aplicações biotecnológicas.

Os principais resultados incluem atividades enzimáticas relevantes (por exemplo, cerca de 1786 U/mL para pectinase e 1359 U/mL para FPase), juntamente com efeitos antifúngicos e nematicidas significativos. O extrato enzimático e o fungo inibiram o crescimento de fungos fitopatogênicos — excedendo 60% de inibição — e reduziram as populações de nematoides em até 75% em 72 horas, demonstrando seu potencial como agentes de biocontrole. Além disso, os extratos enzimáticos foram capazes de decompor poluentes orgânicos persistentes, como atrazina e desetilatrazina, atingindo taxas de remoção de cerca de 75% após 21 dias, o que reduziu significativamente a meia-vida desses contaminantes em comparação com a degradação natural.

Esta pesquisa destaca implicações práticas para a agricultura sustentável, biorremediação e gestão de resíduos. Um ponto forte é o uso de resíduos agroindustriais como substrato e fonte de nutrientes durante a fermentação, o que aumenta a sustentabilidade e a relação custo-benefício do processo. Também é possível observar a versatilidade do extrato enzimático, que envolve aplicações desde o biocontrole até a degradação de poluentes.

No entanto, alguns desafios foram relevantes no processo, como o declínio gradual da atividade enzimática ao longo do tempo e a necessidade de otimização adicional de algumas enzimas, como β-glicosidase e avicelase, para ampliar a escala para aplicações industriais. Como o estudo foi realizado em condições controladas de laboratório, testes de campo em condições reais são essenciais para confirmar a eficácia e a segurança.

Durante este trabalho, observamos lacunas na literatura existente, especificamente em relação à aplicação em larga escala de coquetéis enzimáticos de *P. brevicompactum* e aos potenciais impactos ecotoxicológicos a longo prazo de seu uso. Pesquisas futuras devem ter como objetivo ampliar a escala do processo de fermentação, otimizar ainda mais a produção e a estabilidade dessas enzimas-chave e incluir ensaios de campo abrangentes para avaliar as implicações mais amplas para a agricultura e o meio ambiente. Além disso, explorar como esses extratos brutos enzimáticos interagem com vários substratos e contaminantes pode fornecer *insights* mais valiosos.

Por fim, este trabalho contribui significativamente para a compreensão de *P. brevicompactum* como fonte de enzimas e bioprodutos úteis. Os resultados corroboram o desenvolvimento de soluções mais sustentáveis para biocontrole, biorremediação e conversão de biomassa, abrindo caminho para avanços na biotecnologia verde e na bioeconomia circular.

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