UNIVERSIDADE FEDERAL DO PARANÁ

FERNANDO ENRIQUE ROSAS VEGA

OPTIMIZATION IN THE PRODUCTION AND PURIFICATION OF FUNGAL B-GLUCOAMYLASE FROM THE FUNGUS *ASPERGILLUS ORYZAE* 1808 FOR ITS POTENTIAL APPLICATION IN THE PRETREATMENT OF MILLED CORN FOR CONVERSION INTO BIOETHANOL

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Dissertação apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Luiz Alberto Junior Letti

Coorientadora: Profª. Drª. Luciana Porto de Souza Vandenberghe

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RESUMO

Os biocombustíveis, como o bioetanol, destacam-se como alternativas sustentáveis aos combustíveis fósseis, por serem menos poluentes e derivados de fontes renováveis. A produção de bioetanol a partir de matérias primas amiláceas, como o milho, tem ganhado destaque, especialmente por meio da hidrólise enzimática do amido presente nesses produtos. Nesse processo, enzimas como as alfa-amilases e glucoamilases desempenham um papel crucial, catalisando a quebra do amido em moléculas menores, como a glicose, que posteriormente é fermentada para a produção de bioetanol. Além dessas, outras enzimas, como xilanases, celulases e lipases, também são amplamente utilizadas no tratamento de biomassa para a produção de biocombustíveis. As glucoamilases, em especial, são enzimas responsáveis por catalisar a conversão de oligossacarídeos e dissacarídeos provenientes da hidrólise de compostos amiláceos anteriores em moléculas de glicose. O Brasil, um dos principais produtores de milho do mundo, desempenha um papel importante, gerando uma grande quantidade de produtos amiláceos que poderiam ser aproveitados. O principal objetivo desta pesquisa foi a otimização da produção de glucoamilases fúngicas utilizando o milho moído como substrato, além de avaliar seu potencial hidrolítico para produzir glicose. Este trabalho foi dividido em duas partes; a primeira foi uma revisão bibliográfica focada nas diferentes enzimas que são utilizadas para a produção de biocombustíveis e o efeito que os pré-tratamentos podem ter sobre a matéria orgânica produtora de biocombustíveis. A segunda contém ensaios experimentais buscando a otimização na produção e recuperação de uma glucoamilase proveniente do fungo *Aspergillus oryzae* LPB1808. Depois de analisar os dados obtidos, foi possível produzir uma glucoamilase usando o fungo *Aspergillus oryzae* LPB1808, em uma fermentação submersa (SmF), utilizando produtos agroindustriais (milho moido), e foi possível identificar uma β-glucoamilase com um peso molecular de 60-65 kDa. Após a concentração e pré-purificação por meio de micro e ultrafiltração, foi alcançada uma atividade final de 47,62 U/mL. Além disso, a enzima purificada conseguiu hidrolisar o milho moído, produzindo 0,232 g de glicose por grama de milho moído, indicando que a enzima fúngica apresenta um rendimento de 43,44% em comparação com a versão comercial.

Palavras-chave: Biocombustiveis; enzimas; glucoamilases; glicose; milho moido.

ABSTRACT

Biofuels, such as bioethanol, are emerging as sustainable alternatives to fossil fuels due to their lower environmental impact and their derivation from renewable sources. The production of bioethanol from starch-based raw materials, such as corn, has gained considerable attention, particularly through the enzymatic hydrolysis of starch present in these products. In this process, enzymes such as alpha-amylases and glucoamylases play a crucial role by catalyzing the breakdown of starch into smaller molecules, such as glucose, which is subsequently fermented for bioethanol production. In addition to these enzymes, others such as xylanases, cellulases, and lipases are also widely employed in biomass treatment for biofuel production. Glucoamylases, in particular, are responsible for catalyzing the conversion of oligosaccharides and disaccharides derived from the hydrolysis of starch-based compounds into glucose molecules. Brazil, one of the leading corn producers globally, plays an important role by generating a significant amount of starch-based products that could be utilized. The main objective of this research was to optimize the production of fungal glucoamylases using ground corn as a substrate, as well as to evaluate its hydrolytic potential for glucose production. This work was divided into two parts; the first was a literature review focused on the various enzymes used for biofuel production and the effect that pretreatments can have on biomass, which is crucial for biofuel generation. The second part involved experimental assays aiming to optimize the production and recovery of a glucoamylase derived from the fungus *Aspergillus oryzae* LPB1808. After analyzing the obtained data, it was possible to produce glucoamylase using *Aspergillus oryzae* LPB1808 in submerged fermentation (SmF), utilizing agro-industrial products (milled corn), and a β-glucoamylase with a molecular weight of 60-65 kDa was identified. After concentration and pre-purification via micro and ultrafiltration, a final activity of 47.62 U/mL was achieved. Furthermore, the purified enzyme was able to hydrolyze the milled corn, producing 0.232 g of glucose per gram of milled corn, indicating that the fungal enzyme exhibits an efficiency of 43.44% compared to the commercial version.

Keywords: Biofuels; enzymes; glucoamylases; glucose; milled corn.

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1. INTRODUÇÃO

O aumento da população mundial nas últimas décadas elevou significativamente o consumo global de petróleo e seus derivados. Esse fenômeno, aliado ao esgotamento das reservas de combustíveis fósseis, resultou em um aumento dos preços do petróleo remanescente, destacando a necessidade de explorar alternativas sustentáveis para atender às crescentes demandas energéticas (Aghaei et al., 2022). Nesse contexto, os biocombustíveis, especialmente o bioetanol, apresentam-se como uma opção promissora. Este composto destaca-se por sua facilidade de produção e versatilidade de aplicação, além de possuir propriedades similares às da gasolina ou do diesel, permitindo seu uso misturado com pequenas proporções de gasolina (Zaghetto de Almeida et al., 2024a). Por ser produzido a partir de fontes renováveis, o bioetanol promove uma economia sustentável, reduz as emissões de gases de efeito estufa, é menos poluente e, não compete com outros biocombustíveis provenientes de fontes alimentares, como o biodiesel. No entanto, seu menor conteúdo energético representa uma limitação técnica (Aghaei et al., 2022; Nazli et al., 2024; Waseem et al., 2024).

Atualmente, a maior parte do bioetanol comercial é obtida a partir de produtos agroindustriais, especialmente da biomassa lignocelulósica ou amilácea. As tecnologias de hidrólise desenvolvidas desde o ano 2000 são utilizadas atualmente para produzir cerca de 45 milhões de toneladas de bioetanol anuais, atendendo aproximadamente 40% da demanda global (Aghaei et al., 2022).

A maioria dos subprodutos agroindustriais gerados durante o processamento de matérias-primas para a produção de alimentos ou biocombustíveis é descartada ou eliminada sem um tratamento adequado, embora esses materiais possam ser valiosos se submetidos a processos de pré-tratamento, os quais são projetados para maximizar a eficiência na extração de compostos úteis, como açúcares, para a produção de bioetanol (Fuentes-Cardenas et al., 2022). No entanto, o principal desafio enfrentado por esses processos é o elevado custo dos reagentes, como as enzimas necessárias para seu desenvolvimento. A otimização e redução de custos na produção de enzimas amilolíticas, como alfa-amilases e β-glucoamilases, são essenciais para tornar esses processos mais viáveis e replicáveis (Zaghetto de Almeida et al., 2024a).

A produção enzimática por fermentação tem demonstrado ser uma tecnologia eficaz para a obtenção de enzimas amilolíticas. Entre as principais metodologias estão a fermentação em estado sólido (SSF) e a fermentação em estado líquido ou submersa (SmF) (Reihani et al., 2024; K. K. Valladares-Diestra, Porto de Souza Vandenberghe, & Soccol, 2021; Zaghetto de Almeida et al., 2024a). Esses métodos são ambientalmente amigáveis e rentáveis, permitindo uma melhor separação e recuperação de complexos enzimáticos, além de oferecer controle preciso de parâmetros

fermentativos como pH e temperatura. Essas características tornam essa tecnologia replicável e adaptável para diversas aplicações.

No contexto da produção de biocombustíveis, uma compreensão detalhada dos mecanismos enzimáticos e dos pré-tratamentos da biomassa é essencial para otimizar a conversão de matérias-primas em açúcares fermentáveis. O primeiro capítulo deste trabalho forneceu uma base teórica fundamental ao explorar as enzimas do gênero *Aspergillus* utilizadas na indústria de biocombustíveis e os efeitos dos pré-tratamentos sobre as matérias-primas. Esse conhecimento orientou a pesquisa experimental e contribuiu para a definição das estratégias de otimização empregadas na produção de β-glicoamilase e na seleção dos parâmetros de fermentação (Zaghetto de Almeida et al., 2024a).

As β-glucoamilases são um tipo de enzima amilolítica capaz de hidrolisar ligações α-1,4 e α-1,6 em moléculas de amido, maltose e sacarose, gerando glicose como principal produto. Este açúcar redutor pode ser reutilizado como substrato por leveduras como *Saccharomyces cerevisiae* e *Candida utilis* para a produção de bioetanol, reduzindo seu custo de produção e aumentando seu valor industrial. Diversos microrganismos produzem enzimas amilolíticas por fermentação, destacando-se os fungos filamentosos por sua capacidade de sintetizar múltiplas enzimas, dependendo da fonte de carbono disponível (Abdelwahab, 2020).

O Brasil desempenha um papel-chave na economia agrícola mundial, sendo um dos principais produtores de milho. Estudos de mercado para o ano de 2024 determinaram que a produção de produtos derivados do milho atingiu 130,87 milhões de toneladas, avaliadas em USD 143,62 bilhões. Com um crescimento anual de 3,01%, estima-se que, até 2029, esses produtos derivados possam representar USD 166,57 bilhões (Wu et al., 2024). O milho moído possui um teor de amido próximo a 60%. Esse produto agroindustrial apresenta-se como uma fonte sustentável para a obtenção de açúcares redutores por meio de processos de hidrólise enzimática, contribuindo para a redução do impacto ambiental (Callegaro et al., 2005; Y. Chen et al., 2024; Giacomelli et al., 2012; Massarolo et al., 2019).

O objetivo principal desta pesquisa foi otimizar a produção de β-glucoamilases utilizando milho moído como fonte de carbono em meio fermentativo. Buscou-se formular uma enzima com alta atividade enzimática para aplicação em processos de hidrólise enzimática, destinados à produção de glicose a partir de produtos amiláceos. Essa glicose, por sua vez, será utilizada na produção de bioetanol, promovendo o desenvolvimento de tecnologias mais sustentáveis e eficientes.

2. OBJETIVOS

2.1. Objetivo geral.

Desenvolver uma estratégia de otimização para a produção sustentável de β-glucoamilase por meio da fermentação submersa (SmF), utilizando o milho moído como substrato. Além disso, buscar realizar a separação, purificação, formulação e aplicação da enzima no pré-tratamento de bioamassa, com o propósito de reduzir seu impacto ambiental e converter o amido em glicose, que será utilizada em processos fermentativos para a produção de bioetanol.

2.2. Objetivos especificos.

1. Explorar as principais enzimas produzidas pelo gênero *Aspergillus* e seu papel na otimização da produção de bioetanol e outros biocombustíveis.
2. Analisar o impacto do uso de matérias-primas sustentáveis no desenvolvimento de tecnologias enzimáticas para biocombustíveis, destacando o potencial do milho moído como fonte de carbono.
3. Triagem qualitativa e quantitativa de fungos produtores de β-glucoamilase.
4. Otimização as condições físico-quimicas e composição do meio para a produção de β-glucoamilase pela cepa selecionada em fermentação submersa.
5. Estudo cinético da produção de β-glucoamilase em frascos Erlenmeyer e reator de tanque agitado (STR).
6. Recuperação e purificação parcial de β-glucoamilase por membranas de microfiltração e ultrafiltração.
7. Caracterização de β-glucoamilase e estudos preliminares de estabilidade enzimática.
8. Estudar a formulação do complexo enzimático e avaliar a estabilidade da β-glucoamilase em diferentes condições de armazenamento.
9. Testar o potencial de aplicação do produto enzimático produzido na hidrolise de amido.

3. State of the Art on *Aspergillus* Genus Enzymes in the Biofuel Industry Using Sustainable Raw Materials

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Enzymes produced by the genus *Aspergillus* integrated into the biofuels industry using sustainable raw materials

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Abstract

Renewable energy sources, such as biofuels, represent promising alternatives to reduce dependence on fossil fuels and mitigate climate change. Their production through enzymatic hydrolysis has gained relevance by converting agro-industrial waste into fermentable sugars and residual oils, which are essential for the generation of bioethanol and biodiesel. The fungus *Aspergillus* stands out as a key source of enzymes, including cellulases, xylanases, amylases, and lipases, which are crucial for the breakdown of biomass and oils to produce bioethanol and fatty acid methyl esters (FAME). This review examines the current state of these technologies, highlighting the significance of *Aspergillus* in the conversion of energy-rich waste materials. While the process holds significant potential, it faces challenges such as the high costs associated with enzymatic production and final processing stages. Agro-industrial waste is proposed as an energy resource to support a circular economy, thereby eliminating reliance on non-renewable resources in these processes. Furthermore, advanced pretreatment technologies including biological, physical, and physicochemical methods, as well as the use of ionic liquids are explored to enhance process efficiency. Innovative technologies, such as genetic engineering of *Aspergillus* strains and enzyme encapsulation, promise to optimize sustainable biofuel production by addressing key challenges and advancing this technology towards large-scale implementation

Keywords: Bioethanol; biodiesel; enzymatic hydrolysis; *Aspergillus*; cellulases; xylanases; amylases; lipases; pretreatment.

3.1. Introduction

The increasing global energy demand, projected to grow by 49% between 2007 and 2035 (Cheah et al., 2020) combined with the depletion of oil and coal reserves, underscores the urgent need for sustainable alternatives. Biofuels, particularly bioethanol and biodiesel, have emerged as key solutions to reduce dependence on fossil fuels, which currently account for 80% of global energy consumption (Nazli et al., 2024; Safarian & Unnthorsson, 2018). Within this context, the European Union aims to replace 10% of its automotive fuel with biofuels by 2030, supported by an investment of $93.67 million in second-generation biofuels (Tayyab et al., 2017).

Bioethanol stands out as a prominent alternative fuel due to its high oxygen content, which improves combustion efficiency, and its high-octane number, enabling higher compression ratios in engines (Cheah et al., 2020). Globally, the United States and Brazil lead bioethanol production, contributing over 85% of the 94 billion liters produced annually. Brazil produces 27.1 billion liters from sugarcane, while the United States produces 59.7 billion liters from corn starch. Additionally, countries such as China and India aim to reduce fossil fuel dependency by 20%, with projected bioethanol consumption levels of 3.8 billion and 1.9 billion liters, respectively (J. Chen et al., 2021).

Lignocellulosic biomass, produced in volumes of approximately 1.5×10^{11} tons annually, has the potential to yield up to 442 billion liters of bioethanol if fully utilized (J. Chen et al., 2021; N. Das et al., 2021). However, a significant portion of this resource remains underutilized; for instance, in the United States, 90% of corn residues are repurposed for field preparation (Rezania et al., 2020). Advances in enzymatic hydrolysis, employing xylanases, cellulases, and amylases, have improved the conversion of wheat, corn, and rice residues, as well as municipal solid waste, into fermentable sugars (Gronchi et al., 2019; Nitsos et al., 2017; Pradyawong et al., 2018). Nevertheless, lignin, comprising 17–32% of sugarcane bagasse biomass, poses a significant challenge due to its chemical recalcitrance (Rezania et al., 2020). Pretreatment processes designed to reduce lignin content and enhance cellulose and hemicellulose accessibility are critical, albeit costly (A. K. Kumar & Sharma, 2017; Maurya et al., 2015).

Biodiesel, primarily produced through transesterification with fungal lipases, is derived from vegetable oils and animal fats. Feedstocks include Honne oil (Srikumar et al., 2024), oil palm biomass (Bhatia et al., 2017; Widayat et al., 2017), rapeseed oil (Srikumar et al., 2024), *Koelreuteria integrifolia* oil (H. Zhang et al., 2017), *Jatropha* oil (Nisar et al., 2017; Z. W. Zhou et al., 2021), sunflower oil (Xia et al., 2024), castor oil (Badoei-dalfard et al., 2021; Baskar &

Soumiya, 2016), soybean oil (K. Valladares-Diestra et al., 2020; Zhong et al., 2011), *Pongamia* biodiesel (Perumal & Ilangkumaran, 2017) and algae and microal (Aghabeigi et al., 2023; Roy & Mohanty, 2021) g a e. Unlike bioethanol, biodiesel does not utilize lignocellulosic biomass due to its low lipid content.

Agro-industrial residues represent a key source for biofuel production. Pretreatments (Figure 1), such as physical (milling and grinding) and chemical (alkaline and acidic), are essential for enhancing the efficiency of enzymatic hydrolysis and improving the yields of fermentable sugars (Maurya et al., 2015). However, these processes require specific optimization for each type of biomass, considering its chemical heterogeneity and the generation of inhibitory compounds (A. K. Kumar & Sharma, 2017). This article addresses these strategies within the framework of sustainable technologies for bioethanol and biodiesel production.

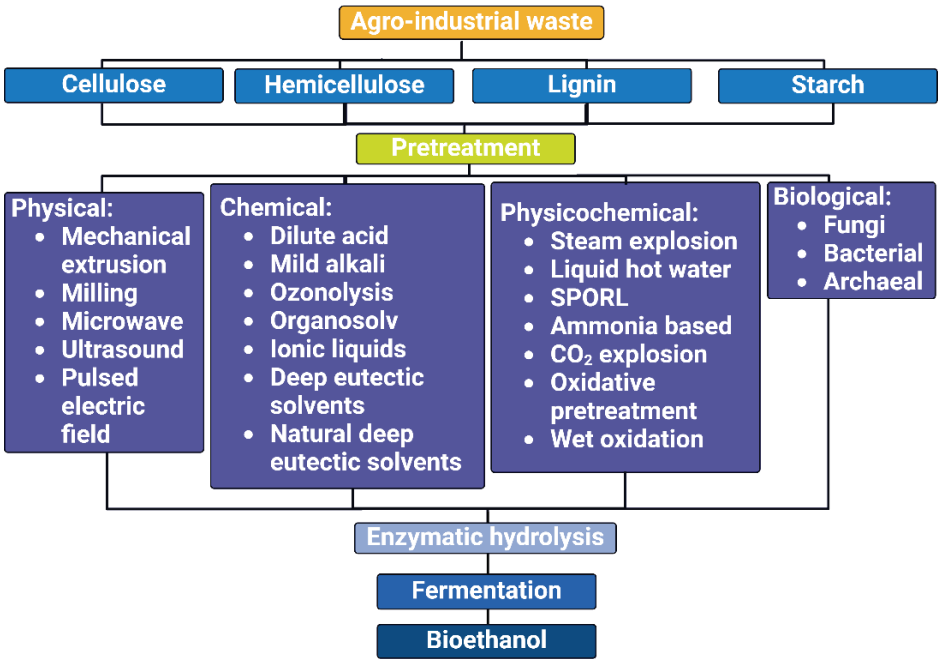

Figure 1. Exploration of different Agroindustrial waste pretreatments, adapted from (Kumar & Sharma, A. K. 2017).

The fundamental principles, advantages, limitations, and performance of these processes in terms of fermentable sugar yields are discussed. This work aims to provide a comprehensive overview of the advances and challenges in biomass pretreatment, offering valuable insights to enhance efficiency and sustainability in biofuel production.

3.2. Renewable sources for biofuels production

In the 21st century, one of the greatest challenges facing humanity is balancing the growing demand for materials, energy, and food with the need to decarbonize the economy to prevent uncontrolled global temperature rise and environmental collapse (Shukla et al., 2007; Wallerstein, 2020). The transition to renewable sources, such as solar, wind, and biomass, is essential for reducing dependence on fossil fuels and greenhouse gas emissions (Usman et al., 2022). Plant biomass, the primary source of carbon on Earth's surface, can be converted into bioproducts such as biofuels, biochemicals, and biomaterials using biorefining techniques that explore the renewability of natural carbon cycles, its sequestration, and subsequent conversion (Louw et al., 2023).

The bioeconomy can be implemented through the utilization of globally available plant biomass resources. First-generation (1G) biorefineries, based on materials such as sugar (sugar beet, sugarcane, or sweet sorghum), starch (corn, cassava, potato), or vegetable oils (rapeseed, soybean, sunflower) (Aristizábal-Marulanda & Cardona Alzate, 2019), are successful, although concerns exist regarding their environmental and social impacts due to competition for resources like water and arable land. Nevertheless, the residues from these production chains (approximately 5 x 10^9 tons annually worldwide) hold significant potential for valorization (Ioannidou et al., 2020; Pleissner et al., 2016). Lignocellulosic biomass is a promising source of second-generation (2G) biofuels, which not only bypasses the food versus fuel debate but also enables the utilization of industrial residues, generating financial returns from materials that would otherwise incur costs for conventional treatment.

Various types of biomass have been evaluated for biofuel production, including invasive plants and small-scale industrial residues such as *Corchorus sp*. (J. Singh et al., 2022), rose flowers (Sahu, 2021), and *Miscanthus giganteus* (Brandt-Talbot et al., 2017). Currently, bioethanol production from such plants is gaining global attention due to their advantages in terms of high biomass yield and sustainability. Several studies have demonstrated that *Miscanthus* can be a viable source for bioethanol production owing to its ability to grow on marginal soils and its low input requirements, making it an attractive option for the biofuel industry. Large-scale projects in Europe and the United States are actively exploring its potential to contribute to the transition towards cleaner and renewable energy sources (Iqbal et al., 2024; Low & Isserman, 2009). For this reason, continuous technological innovation in its processing is essential, as it is crucial to develop solutions that align with local resource availability and requirements, thereby enhancing economic profitability (Vaidyanathan et al., 2019).

As shown in Table 1, numerous emerging biomass sources have been assessed. Bhuyar et al. evaluated the use of *Amorphophallus* sp. tubers, which is a non-edible starchy material generated in tropical regions of the northern part of Thailand. The authors achieved an initial ethanol yield of 8.68 g/L. Although the yield is relatively low, the biomass is considered highly innovative due to its novelty in the biofuel production field (Bhuyar et al., 2022). Similarly, Sahu investigated the exploration of rose flowers waste, which produced 29.5 g/L of ethanol, showing a promising application for these waste streams (Sahu, 2021). More recently, Kabadayi et al. explored the potential of mulberry pomace for bioethanol production by *Hansenispora uvarum*. The authors achieved a 61.3 g/L concentration, further expanding the range of underutilized biomasses for bioethanol production. Also, recent studies have explored the potential of different grasses and, in some contexts, invasive plants for biofuel production (Kabadayı et al., 2024). Iyyappan et al. evaluated elephant grass (*Pennisetum purpureum*) using a biological pretreatment with *Trichoderma reesei* and NiO nanoparticles, achieving an ethanol yield of 14.65 g/L with *Kluyveromyces marxianus* MTCC 1389 (Iyyappan et al., 2023). Wongleang et al studied *T. latifolia* grass with a concentrated acid pretreatment (75% H_3PO_4 at 60 °C for 60 min), resulting in an ethanol yield of 8.9 g/L using Saccharomyces cerevisiae TISTR 5339 (Wongleang et al., 2024). Similarly, Kentucky bluegrass (*Poa pratensis*) was used on a humic acid-assisted alkaline pretreatment, achieving an ethanol yield of 15.3 g/L with *S. cerevisiae* YPH499 (R. Kumar et al., 2022). These findings highlight the promising potential of these grasses as a way for valorizing non-traditional feedstocks, including invasive species, for sustainable bioenergy production.

Adding to that, residues originated from more conventional agricultural commodities, such as rice husks, wheat and barley straw, continue to receive great attention for the development of its production technologies, while a biochemical route with the use of specific enzymes are continually evaluated. Jin et al. reported a bioethanol concentration of up to 108.6 g/L, using *Aspergillus fumigatus* enzymes in alkaline-pretreated rice straw (Jin, Ma, et al., 2020). While Ziaei-Rad et al. achieved 43.1 g/L of bioethanol, with 84.34% of yield, exploring wheat straw hydrolysate under optimized ionic liquid (IL) pretreatment (Ziaei-Rad et al., 2021). Similarly, Duque et al. evaluated and alkaline extrusion pretreatment (7.2% NaOH, 100 °C, 3 h, 120 rpm) followed by enzymatic hydrolysis (10 FPU/g, 20% solids) in a simultaneous saccharification and co-fermentation on barley straw, resulting in 38 g/L ethanol, corresponding to 15.8 g/100 g raw barley straw (Duque et al., 2020).

Despite the advances in recent research, the largest examples of successful biofuel production from plant biomass are located in the USA and Brazil, with massive quantities of 1G bioethanol produced annually in both countries. Corn remains the primary feedstock for bioethanol production in the USA, with enzymatic processes playing a crucial role in the efficient conversion of starch to fermentable sugars (U. Lee et al., 2021). The annual production of bioethanol from corn in the USA reached 57 billion liters in 2019, supported by advanced enzyme technologies for starch hydrolysis and fermentation (U. Lee et al., 2021). In Brazil, sugarcane is the main feedstock, driven by its high energy yield and established infrastructure and policy, resulting in 35.6 billion liters in 2020 (Vandenberghe et al., 2022). However, a significant amount of residual biomass, such as sugarcane bagasse, remains underutilized. This biomass, along with corn residues, such as corncob and corn stover, offers promising potential for 2G bioethanol production (Vandenberghe et al., 2022).

The efficiency of different biofuel production, particularly from 2G sources, hinges on effective pretreatment strategies. These advances are key to optimizing the conversion of lignocellulosic biomass into fermentable sugars, enabling the successful production of biofuel (Bhatia et al., 2020) s. Table 1 also shows different pretreatment technologies recently evaluated under the context of biofuel production. Physical pretreatments, such as milling and crushing, aim size reduction and the increase of the surface area for enzymatic action. Zhou et al. applied a drying process at 105 °C for 2 h and a crushing after that to size reduction to <2 mm. This enabled effective enzymatic hydrolysis and fermentation, reaching 73.2 g/L of bioethanol with the addition of 1% saponin to act as a surfactant in the bioprocess (H. Zhou et al., 2023). On the other hand, chemical methods, such as acid and alkaline pretreatments, are highly effective in biomass fractionation, breaking lignin and hemicellulose bonds; for instance, cassava waste treated with NaOH (0.045 NaOH, 153 °C, 48 min) achieved 93.87% glucose yield (Suriyachai et al., 2024), while two-stage acid-alkaline pretreatments reduced lignin and improved holocellulose accessibility in mango leaves (Tarrsini et al., 2023). Biological pretreatments using fungi like *Pleurotus florida* and *Trichoderma reesei* facilitate lignin degradation and cellulose decrystallization, but under a process that could take more than weeks, which represents the major bottleneck of biological pretreatments (Iyyappan et al., 2023; Ranjithkumar et al., 2022). These approaches, optimized for specific biomasses, must be better understood to be applied on an industrial scale. Further developments in terms of synergies between pretreatment catalysts and enzymes must be developed to significantly enhance the efficiency of biofuel production.

Table 1. Different biomasses and bioprocesses explored for biofuel production.

Biomass	Pretreatment	Biofuel	Strain	Production	Main finding	Ref.
Rice husk	Alkali, HPAC, and alkali-HPAC	Ethanol	*S. cerevisiae*	29.9 g/L (85.4%)	Optimized conditions for enzymatic hydrolysis of rice husk resulted in enhanced ethanol production	(Y. Song et al., 2024)
Rice straw	Alkaline (0.25M Na_2CO_3, 121 °C, 15 min)	Ethanol	*S. tanninophilus*	108.6 g/L	High effective ethanol production using alkaline pretreatment and *A. fumigatus* enzymes.	(Jin, Ma, et al., 2020)
Corn stover	Acid (0.89% H_2SO_4, 125°C, 5 min)	Butanol	*C. saccharobutylicum* DSM 13864	9.02 g/L (97.3%)	A complete ABE biorefinery process based on corn stover was developed, including detoxification and gas stripping	(Hijosa-Valsero et al., 2020)
Pineapple peels	Ultrasonic (5% biomass loading, LSR 20, 15-45 min at 55 °C, 40 khz, 50 W)	Ethanol	*S. cerevisiae*	196.2 g/L	Effective ethanol production from pineapple peels using ultrasonic pretreatment	(Casabar et al., 2020)
Deodar sawdust	Thermochemical pretreatment (0.5 M NaOH solution at 80 °C for 2 h)	Ethanol	*P. stipitis*	14.25 g/L (95.68%)	Optimized conditions significantly increased total reducing sugar concentration	(Raina et al., 2020)
Tobacco waste	Alkaline (10% NaOH, 80 °C, 90 min)	Ethanol	*Mucor hiemalis*	97%	90% desilication reached by Alkaline pretreatment	(Sarbishei et al., 2021)
Acacia wood	Acid (0.05% sulfuric acid, 200 °C, 5 min)	Ethanol	*S. cerevisiae*	4.57 g/L (94.9%)	Soy protein addition improved enzymatic hydrolysis efficiency despite lacking enzymatic activity	(I. Lee & Yu, 2020)
Napier grass	DES (1:4 CHCl/LA, 80 °C, 5 h)	Ethanol	*S. cerevisiae*	86.6%	DES pretreatment resulted in 71% cellulose recovery, 68% delignification, and 87.09% glucose conversion	(Panakkal et al., 2022)
Wheat straw	IL ([TEA][HSO_4], 30 °C, 3 h)	Ethanol	*S. cerevisiae* PTCC 5052	43.1 g/L (84.34%)	Low-cost ionic liquids can effectively pretreat lignocellulosic biomass for high ethanol yields	(Ziaei-Rad et al., 2021)
Sal sawdust	Acid (1.27% hcl, 10% biomass, 22.43 min)	Ethanol	*S. cerevisiae* MTCC-36 and *P. stipitis* NCIM-3498	9.43 g/L (97%)	Sal sawdust from the furniture industry can be effectively transformed into ethanol	(Raina et al., 2020)
Paulownia wood	Two-stage Autohydrolysis (204-222 °C,	Ethanol	*S. cerevisiae* CECT-117	37 g/L (100%)	Sequential two-stage autohydrolysis allows for effective recovery of all fractions of Paulownia wood for ethanol production	(Domínguez et al., 2020)
Pomegranate peel	Hydrothermal (115 °C, 40 min, LSR 10)	Ethanol	*S. cerevisiae* YPH499	12.9 g/L (95.1 %)	Optimized SSF process achieved significant sugar consumption	(Mazaheri et al., 2021)

3.3. Impact of pretreatment on enzymatic production and conversion to biofuels

To assess the impact of pretreatment on organic biomass, it is essential to understand its chemical composition and how these processes can alter its molecular structure. The appropriate selection of the pretreatment process directly depends on the composition of the components present in the organic feedstocks. Each type of biomass exhibits a variable proportion of cellulose, hemicellulose, and lignin, which also determines its behavior in response to different pretreatment methods (Rezania et al., 2020). Optimal pretreatment selection allows for the maximization of fermentable sugar release while minimizing the formation of toxic by-products, ensuring more efficient processes and the production of high-quality biofuels.

Biomass with elevated cellulose concentrations, such as sugarcane bagasse or cellulose residues, requires pretreatments that enhance the accessibility of hydrolytic enzymes such as amylases and cellulases. In this case, physical pretreatments such as milling or crushing, which increase the contact surface area for these enzymes (Prajapati et al., 2020). Alkaline pretreatments can also be employed; the application of solutions such as sodium hydroxide (NaOH) facilitates the partial removal of lignocellulosic fragments, thereby exposing cellulose and improving its conversion to fermentable sugars (Infanzón-Rodríguez et al., 2024).

Biomass rich in hemicellulose, such as sorghum bagasse residues and wheat straw, contains a significant proportion of hemicellulose, requiring specific pretreatments for the release of xylose and other sugars. Acid pretreatments, such as diluted acids (sulfuric acid or nitric acid), are particularly effective for hemicellulose hydrolysis. However, it is important to consider that the generation of by-products like furfural may necessitate a detoxification process to prevent adverse effects on bioethanol quality (Rezania et al., 2020). Ionic liquid pretreatments are an efficient alternative for dissolving hemicellulose without generating inhibitors, resulting in a cleaner process that optimizes the utilization of C5 sugars, increasing the conversion efficiency (Valamonfared et al., 2024).

On the other hand, biomass with contains high levels of lignin, such as rice husks, sawdust, or residues from the wood industry, shows interferent in the digestion of cellulose and hemicellulose. To remove it, alkaline pretreatments, such as sodium hydroxide (NaOH), potassium hydroxide (KOH), or calcium hydroxide ($Ca(OH)_2$), are commonly used due to their ability to significantly reduce lignin, facilitating the accessibility of structural carbohydrates. Lignin reduction improves the digestibility of polymers, promoting the release of fermentable sugars (Chugh et al., 2023; Pardo Cuervo et al., 2024).

Finally, there are balanced organic materials, such as *Miscanthus giganteus*, which present an equilibrium of cellulose, hemicellulose, and lignin, receptive to a variety of pretreatments. For these materials, hybrid pre-treatments are the most suitable, where the combination of acid and alkaline treatments can optimize the extraction of the compounds of interest. This balanced approach improves biomass-to-bioethanol conversion efficiency, maximizing sugar recovery without compromising biofuel quality (Skiba et al., 2017, 2022).

3.3.1. Biological pretreatments with white rot fungi (WRF)

The pretreatment of lignocellulosic biomass using White Rot Fungi (WRF) relies on the action of enzyme-producing fungi, such as laccases, capable of depolymerizing lignin in lignocellulosic materials. This biotechnological approach stands out due to the inherent advantages of enzymes: high affinity for biomass, continuous secretion throughout the mycelium, and enhanced oxygen diffusion, which promotes both mycelial growth and ligninolytic enzymatic activity (A. Tyagi et al., 2025). WRF fungi grow efficiently in solid-state fermentations (SSF) within low-cost bioreactors with simplified designs that do not require mechanical stirring or intensive aeration (A. Gupta et al., 2024). This process is particularly effective at temperatures close to 35°C and under high humidity conditions, making it feasible for implementation in open and cost-effective systems. However, nitrogen availability is a limiting factor, as lignin degradation is favored under low nutrient concentrations and high C/N ratio conditions. Supplementation with specific salts, such as $NaNO_3$ (4%), KCl (1%), and $MgSO_4 \cdot 7H_2O$ (1.4%), has been shown to increase the recovery of substrates and available carbohydrates in cotton stems (Hai et al., 2024). The improvement in enzymatic hydrolysis is attributed to the removal of residual lignin, which reduces the irreversible adsorption of cellulases and favorably alters the physical properties of the substrate. Studies report cellulose recovery rates ranging from 56.74% to 98.4%, and lignin degradation selectivity's between 0.7% and 30.38%, depending on the fungal species and operational conditions (Parmar et al., 2024). Given the high potential of these pretreatments, combinations with other techniques have been explored to optimize efficiency.

Ren et al. employed a microwave-assisted hydrothermal pretreatment combined with fungal fermentation for enzymatic digestion of cereal straw. This approach resulted in a high yield of fermentable sugars and a significantly superior saccharification efficiency with the combined pretreatment (66.28%) compared to the exclusive use of fungi (25.51%) (Ren et al., 2020). Meanwhile, Wang et al. applied a combined process of *Lenzites betulina* C5617 and hot water at high pressure (LHW) to treat poplar wood, achieving a hemicellulose recovery rate of

92.33%, nearly doubling that obtained with LHW alone. This method enhanced glucose yield by 2.66 times compared to thermal-only pretreatment (W. Wang et al., 2012). Ma et al. investigated the combined pretreatment of the ligninolytic fungus *Echinodontium taxodii* and the brown rot fungus *Antrodia sp*. 5898 with diluted acid on water hyacinth biomass. The results demonstrated increases in reducing sugar yields ranging from 1.13 to 2.11 times compared to the isolated acid treatment, highlighting the synergy of the biochemical approach (Ma et al., 2010). Martínez-Patiño et al. performed a sequential pretreatment with *Irpex lacteus* followed by diluted sulfuric acid (2% p/v, 130°C, 90 minutes) on olive biomass, observing a 34% increase in enzymatic efficiency compared to the independent acid pretreatment (Martínez-Patiño et al., 2018). Additionally, Si et al. reported that the combination of ligninolytic bacteria (*Pandoraea sp.* B-6) and diluted acid increased sugar yield by 40.9%, reaching 772.0 mg/g, demonstrating an effective synergistic mechanism (Si et al., 2019). Zhong et al. used White Rot Fungi (WRF) combined with alkaline solutions at ambient temperature to pretreat corn stover, reducing the biological process time to 15 days. This method resulted in a 50.4% increase in glucose yield (271.1 mg/g) compared to the exclusive use of alkaline solutions (Zhong et al., 2011). On the other hand, Shen et al. implemented a synergistic treatment of Na_2CO_3 and the bacterium *Cupriavidus basilensis* B-8 on rice straw, achieving 799.6 mg/g of reducing sugars, a 285% increase compared to Na_2CO_3 alone and 8.15 times compared to raw biomass (Z. Shen et al., 2018). Xie et al. evaluated the effect of a pretreatment of industrial hemp woody core using *Pleurotus eryngii* combined with alkaline and oxidative (A/O) solutions. This approach increased reducing sugar yields by 1.10 to 1.29 times compared to the fungal-only pretreatment, demonstrating a significant improvement in enzymatic saccharification (Xie et al., 2017). Zhuo et al. explored a system based on tetrahydrofuran and water to pre-erosion the surface of corn stover before pretreatment with *Pandoraea sp*. B-6. This approach increased sugar yield by 7.5 times compared to untreated corn stover, attributed to surface modification and substrate porosity, creating a rough and highly accessible structure for enzymes (Zhuo et al., 2018). The bio-coordinated pretreatment using steam explosion (SE) combined with fermentation by *Phellinus baumii* demonstrated increases in sugar yields during enzymatic hydrolysis. The values ranged between 26.3% and 32.3% compared to SE alone, and between 6.5% and 78.1% compared to the exclusive use of WRF. This method achieved a glucose yield of 313.31 g/kg, surpassing 2.88 and 1.32 times the yields obtained with raw biomass and SE alone, respectively (G. Li & Chen, 2014; Ren et al., 2020).

The enzymatic activity resulting from the treatment of lignocellulosic biomass with WRF depends on several factors, including the origin of the enzymes (cellulases, xylanases, and

amylases), biomass loading, temperature, and reaction time. Studies report significant increases in glucose and reducing sugar release after pretreatment with WRF, with increases ranging from 7.5 to 17.6 times compared to untreated materials (W. Sun et al., 2022; A. Tyagi et al., 2025).

Pretreatments of lignocellulosic material using white-rot fungi (WRF), while representing an environmentally friendly option due to their low energy consumption and minimal waste generation, require prolonged timeframes to achieve efficient lignin decomposition, which poses a significant challenge for industrial applicability and scalability of these processes. Additionally, operational costs associated with this technology are increased because of the need to maintain fungal cultures under controlled conditions, directly impacting the economic feasibility of large-scale implementation. Inherent limitations of this biotechnological approach have been identified (Kocaturk et al., 2023; Wan Azelee et al., 2023). WRF exhibits limited growth capacity on species like *Pinus taeda* due to the presence of inhibitory resins. A proposed solution involves a layered approach, where the resins are predegraded using recycled organic solvents through distillation, facilitating fungal action on lignin (A. Tyagi et al., 2025). Pretreating biomass with diluted acid or autohydrolysis aims to remove lignin before fungal action; however, this may reduce the carbohydrates available for fungal consumption and promote delignification by altering the biomass structure (A. Gupta et al., 2024). High viscosity of treated materials limits physical processes like steam explosion or CO_2 supercritical treatment. Using fungi to reduce viscosity, along with CO_2 explosions and organic solvents, helps remove inhibitory molecules generated by lignin and improves the structure for enzymatic hydrolysis (Hai et al., 2024). Fungal mycelium may hinder the binding of fungal cellulolytic enzymes to the substrate, reducing yield. Washing with ionic liquids or polar solvents can cause the biomass to precipitate with an anti-solvent, breaking down mycelium and microcrystalline cellulose structures, thereby increasing the hydrolysis rate (Z. Shen et al., 2018; A. Tyagi et al., 2025).

3.3.2. Alkaline and acid pretreatment

Alkaline pretreatment is widely used on lignocellulosic biomass due to its direct action on the lignocellulosic structure, promoting delignification through the breakdown of α- and β-alkyl and aryl ether bonds. This process induces the deprotonation of phenolic groups in lignin, favoring its depolymerization. It also facilitates the removal of uronic acids and acetyl groups from xylan chains, significantly increasing the substrate's susceptibility to enzymatic hydrolysis (Saroj et al., 2024). Studies related to the production of second-generation biofuels indicate that alkaline pretreatment is a critical initial step. Recent research has evaluated the life cycle of these

solutions, demonstrating the economic feasibility of the process through the recovery and reuse of the alkaline agents used (Rocha et al., 2014).

The impact of alkaline pretreatment on enzymatic activity has been evaluated through optimized processes. Saroj et al. applied sodium hydroxide (0.5 M, 10% p/v) at 121°C for 1 hour, achieving increases in total reducing sugar production. Without pretreatment, the concentrations obtained were 27.59 mg/mL and 34.65 mg/mL for cellulases and xylanases, respectively. After pretreatment, these concentrations increased to 32.13 mg/mL and 40.37 mg/mL. By adjusting the lignocellulosic biomass concentration to 2.5% p/v, maximum values of 34.31 mg/mL and 44.03 mg/mL were reached by incorporating enzymatic combinations (cellulases and xylanases) during 48 hours of hydrolysis (Saroj et al., 2024).

On the other hand, research employing acid pretreatments has increased in relevance in recent years. This type of pretreatment is based on the use of dilute acidic solutions to modify or remove the molecular structures of hemicellulose and lignin present in the biomass, which are key compounds in biofuel production. Recent studies have focused on the application of such pretreatments in large-scale bioethanol production.

Skiba et al. employed nitric acid at two concentrations (1%-4%) to treat Miscanthus pulp, a plant that has gained prominence in the last decade due to its chemical composition and potential for conversion into bioethanol. Low acid concentration was used in a pre-hydrolysis phase to remove dust and interfering organic matter, while higher concentration served as a key pretreatment for the release of cellulose and hemicellulose. Utilizing commercial cellulases, this pretreatment enabled the hydrolysis of up to 90% of the fermentable sugars present, which were then fermented by Saccharomyces cerevisiae Y-1693, achieving a total bioethanol production of 260 L per ton of treated Miscanthus (Skiba et al., 2022). Makarova et al. utilized 4% nitric acid as a pretreatment for two variants of cellulose derived from Miscanthus, employing commercial cellulose at substrate concentrations ranging from 60-90 g/L. This treatment resulted in a 92% hydrolysis of the present cellulose, yielding an approximate production of 30.6 to 40.8 g/L of bioethanol after fermentation with Saccharomyces cerevisiae (Makarova et al., 2017). Finally, Skiba et al. used a 4% nitric acid pretreatment at a solid-to-liquid ratio of 1:20 to treat oat husks. After hydrolysis with cellulases, the total reducing sugar yield after 72 hours of hydrolysis reached 93%, considering an acid-insoluble lignin that did not interfere with enzymatic hydrolysis. Subsequently, the fermentation of these sugars resulted in a production of 0.159 g of bioethanol per gram of treated oat husks, representing a 120% increase compared to processes without pretreatment (Skiba et al., 2017).

Chemical pretreatments, such as acidic and alkaline methods, demonstrate high efficiency in biomass decomposition. However, the by-products generated, including phenolic compounds and residual salts, require proper management, thereby increasing the costs and complexity of the process. Strong acids can produce toxic compounds that must be neutralized, escalating resource consumption and impacting the overall sustainability of the process. For instance, sulfuric acid, commonly used in pretreatment, generates acidic residues or toxic components that are expensive to handle and necessitate additional treatment processes. In terms of costs, acidic and alkaline pretreatments can raise bioethanol production expenses by approximately $0.10–$0.20 USD per liter (Saroj et al., 2024). Sodium hydroxide is the most used alkaline agent, resulting in high operational costs. Additionally, it poses environmental challenges due to the generation of toxic compounds during neutralization and disposal. Implementing chemical recycling systems and strategies to minimize environmental impact is crucial for ensuring the viability of this pretreatment (Prajapati et al., 2020). Although the main goal of the pretreatment is lignin removal, there is a risk of losing soluble sugars and degrading structural components, which not only reduces the total saccharification yield but also generates inhibitory compounds that negatively affect enzymatic activity (Winarsih & Siskawardani, 2020). The chemical heterogeneity of different lignocellulosic biomasses, such as sugarcane bagasse and agricultural residues, complicates the standardization of the process. Pretreatment conditions need to be adjusted for each type of biomass, increasing the complexity of process design (El-Shora et al., 2021). Enzymes like cellulases, xylanases, and β-glucosidases, often derived from fungi such as *Aspergillus sp*., exhibit limited tolerance to by-products generated during alkaline pretreatment, including phenolic compounds and residual lignocellulose, which inhibit their activity and decrease hydrolysis efficiency (Infanzón-Rodríguez et al., 2024). This pretreatment requires elevated temperature conditions and prolonged processing times, significantly increasing energy consumption associated with the process. Implementing alkaline pretreatment at an industrial scale faces both logistical and economic challenges. For successful integration into biorefineries, it is crucial to evaluate not only its technical performance but also its economic sustainability and environmental impact (Chugh et al., 2023).

Chemical pretreatments, such as the use of diluted acids or bases, like sulfuric acid or sodium hydroxide, enable the separation of biomass components. Their impact on the quality of biofuel is significant. Acids, such as nitric acid, are highly effective in solubilizing hemicellulose; however, they can generate inhibitors such as 5-HMF (5-hydroxymethylfurfural) and furfural, which negatively affect fermentative performance and bioethanol quality. Conversely, alkaline agents improve cellulose digestibility and partially remove lignin, but they

may increase the ionic load of the medium, potentially affecting fermentation and the final product quality.

3.3.3. Physicochemical pretreatment

This pretreatment is one of the most reported due to its impact on lignocellulosic biomass, as it not only removes compounds that inhibit enzymatic hydrolysis but also increases the material's porosity. It begins with mechanical grinding to increase the surface area and remove contaminants. The resulting material is then treated with diluted acid solutions (0.5-5% H_2SO_4), which facilitates the denaturation and removal of residual lignin. However, high acid concentrations can generate inhibitory compounds, such as furfural, salts, and phenolic compounds derived from lignin, which reduce enzymatic efficiency. In the context of biorefineries relying on sugar fermentation, this pretreatment has proven to be efficient, reducing the lignin content in the raw material to below 1% and increasing cellulose availability to levels above 82%. Scanning electron microscopy and X-ray spectroscopy analyses have shown progressive structural damage and a reduction in the crystallinity index of cellulose to values below 13.8% (Ranjan et al., 2024).

Ranjan et al. developed a protocol that included grinding the biomass into particles of 2.36 mm, drying at 50 °C for 12 hours, and treating with sulfuric acid (2%) at 55 °C for 24 hours. This approach produced 28.3% p/p fermentable sugars, surpassing the yields obtained from untreated materials (10.2–11.8% p/p). The specific sugar concentrations included 12.5% p/p xylose, 7.4% p/p glucose, 5.4% p/p cellobiose, and 3% p/p arabinose (Ranjan et al., 2024). Nemes et al. performed an acid pretreatment of oat bran by adding 50 mL of an aqueous solution containing 3% sulfuric acid to 10 g of previously ground substrate. The chemical hydrolysis process was carried out for 2 hours at room temperature, followed by controlled drying at 40 °C for 24 hours. Subsequently, enzymatic hydrolysis of the material was conducted using an enzymatic complex produced by the fungus *Aspergillus niger* (ATCC-6275). This process achieved a yield of 44% in total sugars released after 5 days of reaction under controlled conditions, with the release of maltose (10.43 g/g of substrate), glucose (27.27 g/g of substrate), and fructose (6.543 g/g of substrate) (Nemes et al., 2024). Finally, Chugh et al. evaluated the effect of combined autohydrolysis and acid pretreatment on the enzymatic hydrolysis of rice bran. The initial process involved a steam pretreatment at 15 psi for 15 minutes, followed by an acid phase using a 1% H_2SO_4 solution. This approach enabled the release of 368.36 mg of reducing sugars per gram of treated substrate, of which 310.88 mg were glucose, an essential carbohydrate subsequently used for bioethanol production (Chugh et al., 2023).

Utilizing lignocellulosic biomass for biofuel production and high-value compounds faces significant challenges due to its recalcitrant structure. Physical pretreatments, such as grinding, steam explosion, or microwave irradiation, have proven effective in improving biomass accessibility. These methods are energy-intensive, and when non-renewable energy sources are employed, the associated costs increase significantly (Nemes et al., 2024). For instance, grinding is estimated to consume between $0.05 and $0.15 USD per kilogram of processed biomass, thereby elevating operational costs in large-scale processes. Physicochemical pretreatments, such as ammonia fiber explosion (AFEX) or carbon dioxide explosion, offer greater control over the generation of by-products. Nevertheless, their industrial-scale implementation remains costly due to the specialized equipment required. (Ranjan et al., 2024). Many pretreatment methods fail to fully remove lignin or disrupt cellulose crystallinity, which limits enzymatic accessibility for efficient degradation. A critical challenge is the generation of inhibitors such as furfural, HMF (5-hydroxymethylfurfural), or phenolic compounds derived from lignin (Chugh et al., 2023). Ensuring consistent accessibility to cellulose, it is necessary to ensure that the pretreatment facilitates total biomass disruption, allowing all areas of cellulose to be exposed to enzymatic action (Narendra Kumar et al., 2023).

Physicochemical pretreatments combine the accessibility provided by physical pretreatments with the use of chemical agents, such as carbon dioxide explosion or ammonia exposure, to modify or eliminate lignocellulosic structures. Additionally, these processes help to modify the chemical composition of the residues, promoting the conversion of fermentable sugars. However, the formation of degradation products, such as organic acids and phenolic compounds, may decrease the quality of the produced bioethanol, necessitating additional detoxification stages.

3.3.4. Ionic liquid pretreatment

Pretreatment with ionic liquids constitutes a sustainable and efficient alternative for processing lignocellulosic biomass. These solutions, composed of organic cations and anions, exhibit thermochemical stability, low volatility, and a high capacity to dissolve both organic and inorganic molecules. These characteristics allow for improved surface porosity and solubilization of cellulose molecules, facilitating subsequent enzymatic hydrolysis (Acharya et al., 2021). Furthermore, ionic liquids are recyclable and can be diluted in water, reducing their viscosity and optimizing contact with lignocellulosic biomass (Buettner et al., 2022). The mechanism of action of these liquids includes interactions with active sites in lignin molecules, such as the structural units of p-coumaryl alcohol and sinapyl alcohol. However, this approach

requires prior optimization to tailor it to the specific characteristics of the lignocellulosic material to be processed (Y. C. Sun et al., 2019; Sunar et al., 2024).

Sunar et al. developed an ionic liquid composed of isopropyl ethylamine and sulfuric acid in equimolar proportions, which was used as a pretreatment agent for lignocellulosic biomass. A 10% p/v solution of the ionic liquid was prepared, which, after pretreatment, was washed with a water-acetone mixture and subsequently recovered by evaporation. The residual lignin was separated by centrifugation. This method showed a significant positive impact on the enzymatic activity of cellulases and β-glucosidases derived from *Aspergillus sp*., achieving a 76% p/p recovery of fermentable reducing sugars, representing a 470% improvement compared to untreated biomass (16% p/p) (Sunar et al., 2024). Silveira et al. observed a maximum glucose conversion yield of 70.7% when applying a pretreatment with 1-butyl-3-methylimidazolium acetate in combination with supercritical carbon dioxide and ethanol for 12 hours at 180 °C, using sugarcane bagasse (SCB) as the substrate (Silveira et al., 2015). Brandt-Talbot et al. reported a 77% sugar release through enzymatic saccharification after pretreating *Miscanthus giganteus* with ionic liquids at 120 °C for 480 minutes (Brandt-Talbot et al., 2017).

Ionic liquids, while a promising technology due to their ability to efficiently and selectively dissolve biomass, face significant economic challenges. The synthesis and recycling of ionic liquids remain expensive, with costs ranging from $20 to $30 per kilogram of product, hindering their large-scale implementation. Although their efficiency in biomass dissolution surpasses that of other pretreatment methods, their cost remains a substantial barrier to scalability. (Sunar et al., 2024). The high cost and limited recyclability of ionic reagents remain major barriers to scaling ionic liquid pretreatments. Although these liquids have been shown to be recyclable, their recycling efficiency depends on operational conditions and the type of lignocellulosic biomass used (Acharya et al., 2021). Ionic liquid pretreatment can alter the biomass structure, although lignin removal is the goal, the integrity of cellulose and hemicellulose may also be affected, potentially compromising the performance of subsequent processes (Awodi et al., 2022). Ionic liquids may directly interfere with enzymatic activity; it has been observed that these compounds can alter the conformation of enzyme complexes, affecting their ability to recognize and degrade glucosidic linkages in cellulose (Beltagy et al., 2022). Combining ionic liquid pretreatment with fermentation systems, such as bioethanol production or biotechnological products, requires careful design. The toxicity and accumulation of inhibitory by-products generated during pretreatment must also be considered, as they may

interfere with the recovery and purification of final products (K. K. Valladares-Diestra, Porto de Souza Vandenberghe, Zevallos Torres, et al., 2021).

Ionic liquid pretreatments, such as 1-butyl-3-methylimidazolium chloride, have emerged as a promising technology due to their ability to dissolve lignin and hemicellulose without generating significant amounts of inhibitory compounds. Their influence on biofuel quality is notable. One of the main advantages of this pretreatment is that it provides a highly accessible substrate for enzymes, maximizing the production of fermentable sugars and minimizing secondary waste. However, residues of ionic liquids in the biomass can affect fermentation or biofuel quality if not completely removed.

Figure 2 summarizes the metabolic pathways followed by lignocellulosic biomass, microalgal biomass, and vegetable or waste oils to be converted into biofuels.

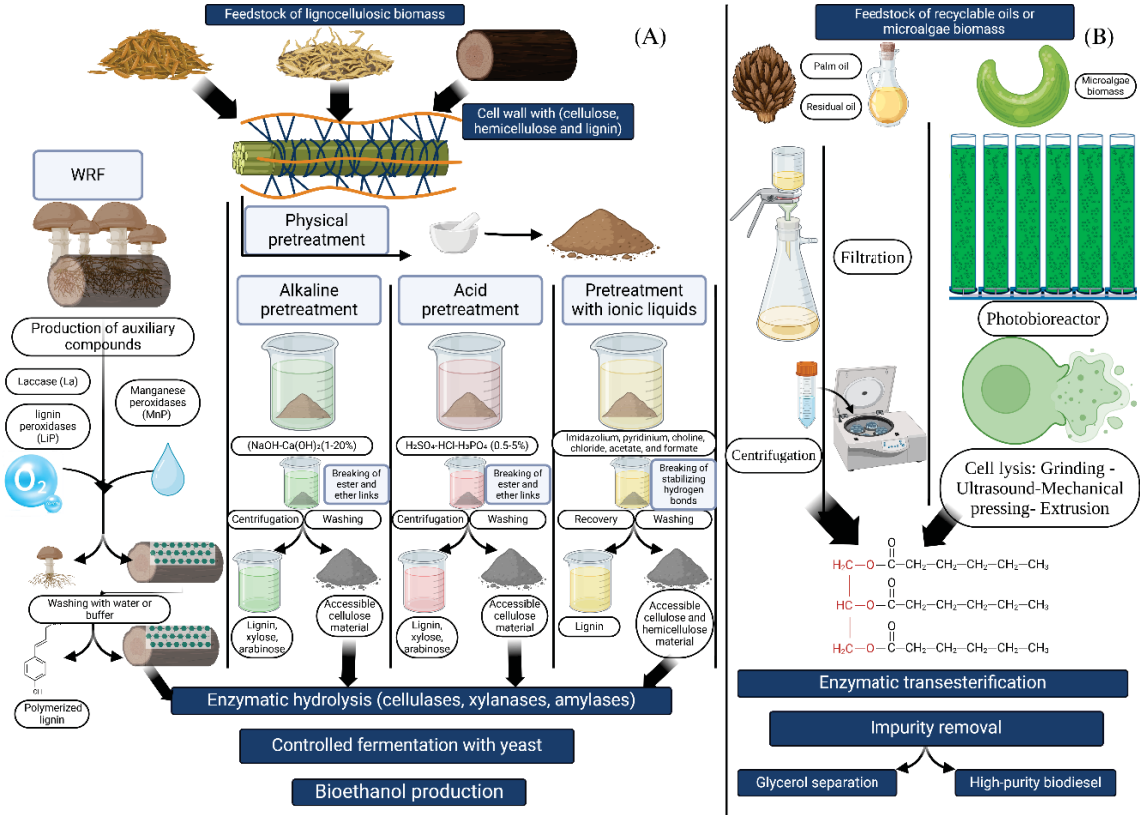

Figure 2. Pretreatments for bioethanol and biodiesel: From biomass to energy. (A): Pretreatments used in lignocellulosic biomass for bioethanol production. (B) Pretreatments in oils, fats, and microalgae biomass for biodiesel production.

3.3.5. Pretreatment for enzymatic transesterification

The release of natural lipids produced by microalgae can be achieved through mechanical, chemical, or combined methods, such as cell rupture by ultrasound or the use of solvents like n-hexane or ethanol. These strategies enhance the release of intracellular lipids, increase the availability of triglycerides and fatty acids for enzymatic reactions, and remove non-lipid compounds, such as proteins and carbohydrates, which may interfere with the enzymatic transesterification process (Tien Thanh et al., 2022).

Raw oils extracted from microalgae often contain impurities such as phospholipids and heavy metals generated during cell lysis or acquired by the microalgae during metabolism. Enzymatic degumming, performed through treatments with hot water or acids, allows for the removal of these impurities. Additionally, oil purification by adsorption with activated carbon is effective in removing trace metals, ensuring an efficient transesterification process (Passos et al., 2024). The presence of water in microalgal biomass or extracted oils can interfere with lipase activity during transesterification. To mitigate this effect, drying techniques at controlled temperatures or the use of dehydrating agents such as calcium chloride ($CaCl_2$), silica gel, magnesium anhydride ($MgSO_4$), or aluminum oxide (Al_2O_3) are employed. These dehydrating agents do not interfere with enzymatic reactions and ensure effective conversion (ShenavaeiZare et al., 2021).

Tien Thanh et al. demonstrated that the release of intracellular lipids from microalgae, such as *Chlorella pyrenoidosa*, is essential for transesterification. High moisture content in the biomass (90%) significantly reduced biodiesel yield (10.3%). However, after subjecting the biomass to a purification process with ethanol and drying at 60 °C for 24 hours, the yield increased to 91.4% (Tien Thanh et al., 2022). Passos et al. reported that degumming with a phospholipase cocktail is a key step in pretreating soybean oil for transesterification using lipases from *Aspergillus niger*. A biodiesel yield of 97% was achieved under optimal conditions: an ethanol-to-oil ratio of 4.48:1, a moisture content of 3.41%, and a lipase concentration of 2.43% (Passos et al., 2024). Finally, ShenavaeiZare et al. investigated the importance of dehydration in oils extracted from halophytic plants such as *Salicornia persica* and safflower. They employed calcium chloride as a catalyst in a system with a methanol-to-oil concentration of 12.9% and 14% calcium chloride, achieving a 97.01% biodiesel yield after 3 hours of reaction (ShenavaeiZare et al., 2021).

The only way to rigorously assess the impact that pretreatments may have on fungal enzyme hydrolysis and bioethanol production is through the optimization of these processes

(Infanzón-Rodríguez et al., 2024). To this end, a compilation of research data is presented in Table 2, which outlines studies on physical (Kitson-Hytey et al., 2024), chemical (Valamonfared et al., 2024), and ionic (Brandt-Talbot et al., 2017) pretreatments that influence the release of reducing sugars from various sources of lignocellulosic biomass, such as rice straw, soy hulls, and banana peels, among others, after being hydrolyzed using enzymes derived from *Aspergillus*. The considered pretreatments include the use of alkaline (NaOH) (106), acidic (H_2SO_4) (John et al., 2020), and ionic solutions (K. Valladares-Diestra et al., 2020). In this context, the amount of sugars released (expressed in mg/g of biomass) is presented, comparing the concentrations obtained from different treatments and highlighting the most significant effects on the efficiency of enzymatic hydrolysis. Furthermore, the impact of different pretreatments on the final bioethanol production is discussed, comparing the bioethanol production yields (in g/L) obtained after fermentation with various *Saccharomyces cerevisiae* (Infanzón-Rodríguez et al., 2024) and *Candida shehatae* (Prajapati et al., 2020) strains. Yields are analyzed under standard fermentation conditions (pH 4.5-5.5, 30 °C).

The pretreatment of oils and microalgae biomass, while representing an attractive source of lipids for biodiesel production, also face significant economic and environmental challenges. The use of organic solvents for lipid extraction can generate waste that is difficult to manage and contributes to environmental contamination if appropriate recovery systems are not implemented. Moreover, the high cost of organic solvents increases operational expenses associated with extraction processes, further complicating their large-scale application (Passos et al., 2024). Phospholipids, free fatty acids, and polar compounds in crude oils obtained from agro-industrial by-products or unrefined oils are considered impurities that interfere with the process. Although enzymatic or chemical degumming has proven effective in reducing these impurities, achieving complete removal without affecting essential oil components remains a challenge (Wei et al., 2023). Treatment of oils through neutralization or drying can alter the chemical properties of the substrate, impacting the three-dimensional structure of triglycerides and, consequently, the activity of fungal lipases (Liu et al., 2024). Water is a key factor in enzymatic transesterification (Amoah et al., 2017). Drying at high temperatures may induce triglyceride hydrolysis, reducing biodiesel yield, therefore, a low-temperature drying process tailored to the oil characteristics is required, which must be optimized for each type of oil used in the process (Zulfiqar et al., 2021). The use of low-quality oils, such as residual oils or those from lignocellulosic materials, presents additional challenges. These oils often contain high levels of metallic contaminants or enzymatic inhibitors, necessitating additional purification

processes that increase costs and complicate the scaling of the process to industrial levels (Guldhe et al., 2016).

Table 2. Impact of pretreatments on reducing sugar release and bioethanol production from lignocellulosic biomass and *Saccharomyces cerevisiae* fermentation.

Pretreatment	Biomass	Enzyme	Microorganism	Sugar produced	Production of bioethanol	References
Physical-alkaline	Corncobs	Cellulase: Endoglucanase Exoglucanase β-glucosidase	*Aspergillus niger*	128.20 g/L	6.4 g/L	(Winarsih & Siskawardani, 2020)
	Sugarcane bagasse	cellulases-hemicellulases: β-glucosidase endo-β-glucanase β-xylosidase endo-β-xylanase	*Aspergillus tubingensis* NKBP-55	20 g/L	15.54 g/L*	(Prajapati et al., 2020)
	Pongamia	Cellulases: endo-β-d-glucanase exo-β-d-glucanase β-glucosidase	*Aspergillus calidoustus*	-	4.4 g/gds	(Narendra Kumar et al., 2023)
	Wood waste			-	2.2 g/gds	
	Rice straw	Cellulases: CMCase β-glucosidase)	*A. fumigatus*	557.8 mg/gds	9.45 g/L	(Jin, Song, et al., 2020)
Physicochemical (acid-alkaline)	Sugarcane bagasse	Cellulases	*Aspergillus niger* ITV02	49 g/L	22.4 g/L	(Infanzón-Rodríguez et al., 2024)
	Wheat bran and sawdust	β-glucanase	*Aspergillus niger* EG-RE (MW390925.1)	37.5 g/gds	12 g/L	(El-Shora et al., 2021)
Physical-		Celullases-	*Aspergillus*		37.63 g/L-	(Chugh et

acidic	Rice bran	Amylases-Xilanase:	*niger* P-19	468 mg/gds	0.41 g/gds	al., 2023)

		β-glucosidase CMCase Xylanase α-amylase β-glucoamylase				
	Chlorella sorokiniana, *Tetraselmis sp.* *Skeletonema sp.*	α-amylase β-glucoamylase	*Aspergillus oryzae*-*Aspergillus niger*	464 mg/gds 420 mg/gds 425 mg/gds	16.512 g/L 7.92 g/L 9.792 g/L	(Constantino et al., 2021)
	Banana peel	Cellulase	*Aspergillus niger*	-	4.24 g/L	(John et al., 2020)
	Wheat straw	Cellulases	*Aspergillus fumigatus*	889.1 mg/gds	21.88 g/L	(Valamonfared et al., 2024)
	Watermelon peels	Cellulases, α-amylase,	*Aspergillus niger*	-	57 g/L	(Olawale et al., 2021)
	Coffee pulp	Cellulase	*Aspergillus niger* MT328516	741 mg/gds	71.39 mg/mL	(Abeleda et al., 2020; Aggarwal et al., 2019; S. Wang et al., 2016)
Physical	Wheat bran		*Aspergillus flavus* MT328429	633 mg/gds	11.73 mg/mL	
	Yam peels banana peels	Cellulases, α-amylase Xylase	*Aspergillus niger*	69.7 mg/gds 65.2 mg/gds	31.86 g/L 22.72 g/L	(Kitson-Hytey et al., 2024)
	Cassava starch	α-amylase β-glucoamylase	*Aspergillus awamori*	-	0.46 g/gds	(Roble et al., 2020)
Physical-ionic	Sugarcane bagasse	Xilanases	*Aspergillus niger*	297 mg glucose/gds 236 mg xilose/gds	10 g/L-0.42 g/gds	(Valladares-Diestra, K.K., et al., 2021)

Mango seed starch	α-amylase	*Aspergillus niger*	848 mg/gds	31.40 g/L	(Awodi et al., 2022)
Starch	alfa-amylasa	*Aspergillus flavus* AUMC10636	28.85 g/L	14.74 g/gds	(Beltagy et al., 2022)

* Fermentation with *Candida shehatae* NCIM 3501 of reducing sugars released after enzymatic hydrolysis with cellulases and hemicellulases from the fungus *Aspergillus tubingensis* NKBP-55

On another note, to evaluate the impact of pretreatments on the lipase activity of *Aspergillus* fungi, enzymatic transesterification is carried out for biodiesel production, with results summarized in Table 3. This table compares the lipase activities of various *Aspergillus* strains, considering that the raw materials used come from different sources, such as soybean oil, palm oil, Jatropha oil, and microalgal biomass. This implies that their composition requires adequate characterization for pretreatment standardization. Enzymatic performance is expressed as the percentage (%) of conversion of fatty acids to biodiesel. Moreover, the incubation conditions (temperature, pH, and time) that optimize biodiesel production are highlighted. This table summarizes the impact of various fermentation parameters, such as pH, temperature, substrate concentration, and incubation time, on biodiesel production using *Aspergillus* lipases. Additionally, comparisons between free and immobilized lipases are included, emphasizing the improvement in the conversion of oils to biodiesel under optimal fermentation conditions.

Table 3. Influence of pretreatments on the yield percentage of enzymatic transesterification catalyzed by *Aspergillus* lipases.

Pretreatment	**Raw material**	**Enzyme**	**Producer**	**Yield (%)**	**Conditions**	**Reference**
Gravitational settling	*Scenedesmus obliquus*	Immobilized whole-cell lipase	*Aspergillus niger*	90.8	36 h, 35 °C, 5:1 ratio (methanol: oil)	(Guldhe et al., 2016)
Natural settling followed by lyophilization	*Chlamydomonas* sp. JSC4	Immobilized whole cell lipase	*Aspergillus oryzae*	97	32 h, 30 °C, 7:1 ratio (methanol: oil)	(Amoah et al., 2017)

Filtration	Waste cooking oil	Co-immobilized lipases	*Aspergillus oryzae*	98.5	24 h, 40 °C, 4:1 ratio (methanol: oil)	(Wei et al., 2023)
	Waste cooking oil	Lipase (1,3-specific)	*Aspergillus oryzae*	98.5	9 h, 40 °C, 4:1 ratio (methanol: oil)	(Liu et al., 2024)
Soxhlet extraction.	*Jatropha curcas* seed oil	Lipase immobilized with TiO2	*Aspergillus niger*	92	30 h, 37 °C, 6:1 ratio (metanol:oil)	(Zulfiqar et al., 2021)

3.3.6. Global impact of biofuel production

Lignocellulosic biomass pretreatments can generate various negative environmental impacts. Specifically, chemical pretreatments, both acidic and alkaline, may lead to the formation of problematic by-products. Organic acids such as acetic acid, furfural, and levulinic acid can form during acidic pretreatments, and these compounds are toxic to the microorganisms used in fermentation, reducing biomass conversion efficiency (Chugh et al., 2023; Ranjan et al., 2024). In alkaline pretreatments, compounds such as sodium hydroxide or potassium hydroxide generate alkaline residues that require proper management to avoid soil and groundwater contamination (Rocha et al., 2014; Saroj et al., 2024). Furthermore, ionic liquid pretreatments, despite their promise of greater sustainability, often require corrosive solvents like hydrochloric acid or sodium hydroxide for residue neutralization, posing risks to both human health and ecosystems. Additionally, ionic liquids are challenging to recover and recycle, exacerbating environmental impacts if not properly managed (Silveira et al., 2015; Sunar et al., 2024). The energy-intensive nature of enzymatic processes presents a significant environmental challenge, the use of enzymes for biomass conversion requires controlled operating conditions, often involving elevated temperatures and pressures, leading to considerable energy consumption and a larger carbon footprint, especially when energy sources are derived from fossil fuels, emissions associated with biocatalyst production include greenhouse gases such as carbon dioxide and methane, primarily stemming from fermentation processes used for enzyme production (Nemes et al., 2024). Moreover, large-scale production of biocatalysts generates industrial waste, including unconsumed substrates, microbial biomass, and intermediate products, which require treatment to prevent accumulation and contamination (Buettner et al., 2022). A potential solution to mitigate these negative impacts involves implementing circular economy processes and

optimizing production pathways, technologies for recovering and recycling ionic liquids and solvents can minimize waste generation, improving pretreatment processes through the use of more efficient catalysts or biocatalysts capable of operating at lower temperatures can reduce energy consumption (Berwian et al., 2024; Mignogna et al., 2024). Focusing on reducing industrial waste through the valorization of by-products, such as organic acids or microbial biomass, contributes to the overall sustainability of the process.

White-rot fungi pretreatments, while promising in terms of sustainability and low environmental impact, face limitations at a large scale due to the slow pace of biological reactions. For instance, fungal fermentation processes require extended timeframes (7 to 10 days), whereas conventional technologies such as acid hydrolysis can be completed in just 4 to 6 hours, this discrepancy negatively affects production timelines and cost competitiveness, particularly when considering industrial-scale operating costs, which range between $0.50 and $1.00 per gallon of bioethanol produced (Hai et al., 2024; Martínez-Patiño et al., 2018). Physical and chemical pretreatments, such as acid and alkaline hydrolysis, are widely used in the bioethanol industry due to their speed and optimization for large-scale production, with operational costs ranging between $0.30 and $0.50 per gallon of bioethanol, acid hydrolysis typically requires large amounts of thermal energy, increasing operational costs and contributing to a higher carbon footprint, with CO_2 emissions exceeding 1 ton per 1,000 liters of bioethanol produced (Miranda et al., 2019; Wei et al., 2023). In comparison, ionic liquid pretreatments, although more efficient in terms of selectivity and fermentable sugar generation, require the use of highly corrosive solvents, this increases operational costs, elevating bioethanol production costs to approximately $1.50 per gallon due to additional risks and solvent handling expenses. Transesterification for biodiesel production, one of the most used methods at an industrial scale, demonstrates greater operational efficiency in terms of time and costs, with a biodiesel production cost estimated at approximately $2.50 per gallon (Buettner et al., 2022).

Emerging technologies such as ionic liquid pretreatments have yet to achieve competitive cost standards compared to these mature technologies. Although biomass conversion efficiency is higher with advanced pretreatments, the costs of solvents, challenges in recycling, and potential material losses remain significant economic barriers, the potential advantages of emerging technologies, their scalability remains uncertain. For instance, while the use of commercial enzymes for bioethanol production an additional cost of approximately $0.30 per gallon, experimental-phase technologies may require up to three times more in operational costs due to the lack of optimization in biocatalyst use (Passos et al., 2024; Roble et al., 2020).

For emerging technologies to become competitive, it is essential to reduce these operational costs, improve energy efficiency, and overcome obstacles related to the safety and management of by-products, the effective integration of these technologies into existing industrial infrastructures will be key to ensuring a smooth transition toward more sustainable and economically viable processes.

3.4. Enzymes produced by *Aspergillus*

3.4.1. Amylases

Amylases are classified according to their mechanism of action into four main groups: endoamylases, exoamylases, branching enzymes, and transferases. Endoamylases, such as α-amylase, hydrolyze the α-1,4 glycosidic bonds randomly within starch chains, whereas exoamylases cleave the bonds at the chain ends, with β-amylases acting solely on α-1,4 glycosidic bonds and amyloglucosidase (glucoamylase) also acting on α-1,6 glycosidic bonds (Van Der Maarel et al., 2002).

Alpha-amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) are extracellular endoenzymes that hydrolyze the internal α-1,4 glycosidic bonds of starch chains (Abeleda et al., 2020; Aggarwal et al., 2019; S. Wang et al., 2016) These enzymes breaks down long-chain carbohydrates by acting at random sites along the starch chain, ultimately producing maltotriose and maltose from amylose, or maltose, glucose, and limit dextrin from amylopectin. However, these enzymes do not have the ability to break terminal glucose residues or α-1,6 linkages. One of their characteristics is that α-amylases tend to act faster than β-amylases because they can act anywhere on the substrate (de Souza & e Magalhães, 2010; Tiwari et al., 2015).

The two-dimensional structure of typical alpha-amylases and their putative homologs mostly involves three basic domains, denoted as A, B, and C. Domain A is defined as the (β/α) 8 domains with its catalytic residues, where glutamic acid acts as a proton donor and aspartic acid acts as a nucleophile at its catalytic sites (Paul et al., 2021). The B domain is a long loop protruding between β3 strand and α3 helix, while the C domain has an antiparallel beta-sandwich structure consisting of eight strands (Q. Zhang et al., 2017). As they are calcium-dependent, the absence of these ions could affect the structure, function, and stability of the enzyme, potentially leading to irreversible inactivation (Xian et al., 2015). These ions are responsible for maintaining the protein structure in its correct conformation, thereby enabling the enzyme to withstand thermal inactivation. There are also reports suggesting that calcium ions play a primarily structural role, as the catalytic sites are distant from the calcium-binding sites (Liao et al., 2019).

β-amylases (EC 3.2.1.2) are exoenzymes belonging to the GH14 family of hydrolases, capable of cleaving α-1,4 glycosidic bonds at the non-reducing ends of starch, producing β-maltose and β-limit dextrins (Duan et al., 2021; Zhu et al., 2011). When amylopectin is subjected to the action of this enzyme, the glucan chains α-1,4 of the highly branched molecule are trimmed from the chain end toward the α-1,6 branching points (Taniguchi & Honnda, 2009). The term beta refers to the initial anomeric configuration of the released free sugar group and not to the configuration of the hydrolyzed bond (Eck, 2013). In plants, β-amylases are associated with fruit development, ripening (hydrolyzing stored starch in fruits to maltose, giving ripe fruits their sweet taste), seed germination, and response to abiotic stress (Mondal et al., 2022). Plants such as sweet potato, soybean, and barley are used as sources of β-amylase. However, due to their disadvantages, such as high production costs and low stability during storage, they are not ideal for industrial processes. The most viable alternative is the use of microbial β-amylases because their production is not affected by the season or climate, they undergo simpler processing, have uniform nature, are more stable, and are easier to handle (Duan et al., 2021). Their structure consists of two peptide domains, a large A domain composed of amino acids from 1 to 417, and a B domain comprising amino acids from 418 to 516. Domain A exhibits a barrel structure (β/α) 8, which is similar in both plant and microbial β-amylases. This structure resembles a pocket where α helices and β sheets associate to form the catalytic site (Nag et al., 2021).

Glucoamylases (EC 3.2.1.3) are exoenzymes that catalyze the hydrolysis of α-1,4- and α-1,6-glucosidic bonds, with a lower efficiency for α-1,6 cleavage, releasing β-D-glucose from the non-reducing ends of starch, as well as related polysaccharides and oligosaccharides (Xu et al., 2016; Zhu et al., 2011). Additionally, they can hydrolyze, at a slower rate, nearly all α-glycosidic bonds, including α, β-(1,1), α-(1,2), and rare α-(1,3) bonds, except for α, α-trehalose (P. Kumar & Satyanarayana, 2009). These enzymes have an invertase reaction mechanism, as there is a transfer of protons from a general acid catalyst to the glycosidic oxygen, followed by the nucleophilic attack of a deprotonated water molecule, assisted by a general basic catalyst (Marín-Navarro & Polaina, 2011). Glucoamylase is the primary hydrolytic enzyme used for saccharification in the fermentation process to produce substances with glucose (Zong et al., 2022). Glucoamylases are hydrolytic enzymes of particular importance in the food and pharmaceutical industries (Zambare, 2010). From a structural standpoint and according to their origin, glucoamylases can be classified into five types (I, II, III, IV, and V). Glucoamylases obtained from filamentous fungi can be of type I or II. On the other hand, those from yeasts can be of type III and IV, while glucoamylases from prokaryotes are of type V. Type I glucoamylases (Gas) contain a catalytic domain (CD) at the N-terminal end linked to a carbohydrate-binding

module (CBM) 20 at the C-terminal end. Type II GAs have a CBM21 attached to the catalytic domain; the β-sandwich structure of the CBM21 domains is similar to that of CBM20, although the CBM21 domains present in type II GAs are always located at the N-terminal end and have two starch-binding sites (Wayllace et al., 2023).

In filamentous fungi, differences have been observed in glucoamylases regarding their molecular mass, amino acid sequence, protein stability, glycosylation percentage, and within and outside the starch-binding site. Up to six different forms of glucoamylases have been found. Most of these glucoamylases are multidomain enzymes with a catalytic N-terminal domain and a C-terminal domain containing starch binding sites. However, exceptions include *Rhizopus oryzae, Aspergillus niger, Aspergillus oryzae, and Aspergillus flavus* NSH9, whose glucoamylase lacks the starch-binding domain structure (Karim & Tasnim, 2018). In type III glucoamylases, the catalytic domain (CD) is not associated with any non-CD or variable region. These enzymes lack additional domains commonly found in other types of glucoamylases. Despite not having a CBM, there are reports of their ability to bind to starch. It is possible that the enzyme uses alternative regions within its CD to interact with starch and carry out its hydrolytic activity. Type V glucoamylases have a catalytic domain attached to an N-terminal region composed of 18 antiparallel β-strands arranged in β-sheets of a super β-sandwich structure, which would confer thermal stability to the enzyme. The C-terminal catalytic domain is a barrel (α/α)6, lacking the peripheral subdomain of eukaryotic glucoamylases. The binding region between the N-terminal region and the catalytic domain is common to all proteins in the GH15 family of prokaryotes (Wayllace et al., 2023).

3.4.2. Cellulases

Cellulases are enzymes that break down β-glycosidic bonds in carbohydrate molecules. Efficient cellulose hydrolysis requires the coordinated action of a cellulase enzyme complex, which consists of three main types of enzymes: endoglucanase (endo-β-1,4-D-glucan glucanohydrolase, EC 3.2.1.4), exoglucanase or cellobiohydrolase (β-1,4-D-glucan cellobiohydrolase, EC 3.2.1.91), and β-glucosidase or cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21) (Ilić et al., 2023).

These three enzymes work together to achieve complete cellulose hydrolysis. Endoglucanase primarily targets the amorphous regions of cellulose, where it randomly breaks internal bonds, creating new chain ends that can be attacked by the other enzymes. This enzyme shows the highest activity against soluble cellulose or acid-treated amorphous cellulose. Exoglucanase, on the other hand, cleaves cellulose chains from the reducing or non-reducing

ends, producing glucose or cellobiose units. Finally, β-glucosidase breaks down cellobiose into glucose, but it does not act on either amorphous or crystalline cellulose.

Although the exact mechanism is not fully understood, early stages of cellulose hydrolysis involve the fragmentation of cellulose aggregates into short fibers, a process known as amorphogenesis, which occurs before any detectable release of reducing sugars (Jayasekara et al., 2019).

3.4.3. Xilanases

Xylanases (EC 3.2.1.8) belong to the class of hydrolase enzymes and are responsible for the breakdown of xylan into xylose and xylan oligosaccharides (Y. Chen et al., 2022). Xylan is a predominant polymer in hemicellulose, which constitutes the plant cell wall, being the second most abundant natural polymer on Earth after cellulose, accounting for approximately 33% of lignocellulosic biomass (Knob et al., 2013). Xylanase includes several subclasses, such as endo-1,4-β-D-xylanases (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), ferulic acid esterase (EC 3.1.1.73), α-glucuronidase (EC 3.2.1.139), and p-coumaric acid esterase (EC 3.1.1.B10), which primarily act on the β-1,4 linkages present in the xylan structure (Lopes et al., 2021; Mano et al., 2018; R. D. Singh et al., 2019; Vázquez et al., 2000). Among these hydrolytic enzymes, endo-xylanases represent the largest group and are currently applied in four main areas: (i) degradation of agricultural residues; (ii) enzymatic treatment of animal feed; (iii) production of dissolved pulps for cellulose manufacturing used in rayon production; and (iv) pre-treatment of kraft pulp or fiber, promoting lignin removal and altering paper properties (Kaushal et al., 2021).

Xylanases with high specific activity play a crucial role in the biodegradation of hemicellulose, making them of significant importance to industry. The biological properties of these enzymes are primarily determined by the active amino acids located in their active sites. According to the CAZy database (Carbohydrate-Active Enzymes., 2024)), xylanases (EC 3.2.1.8) are grouped into several families of glycoside hydrolases (GH), such as GH5, GH8, GH10, GH11, GH30, GH43, GH51, and GH98. However, most research has focused on xylanases from the GH10 and GH11 families. Among these, xylanases from the GH11 family are considered true xylanases, characterized by their low molecular weight and the presence of a conserved β-jellyroll structure (Kaushal et al., 2021; D. Tyagi & Sharma, 2021). These enzymes offer several advantages, such as high catalytic efficiency, strict substrate specificity, and stability over a wide range of pH and temperature conditions, making them valuable for

industrial applications in areas such as food production, animal feed, paper pulp processing, and juice clarification (Malhotra & Chapadgaonkar, 2018).

The protein structure of xylanase consists of specific functional units that are associated with the arrangement of active amino acids and their interactions with various biochemical properties of the enzyme. Within the active site, the amino acids play a crucial role in substrate recognition, catalysis, and product release. Hydrogen bonds and stacking interactions are essential forces that facilitate ligand binding, and the active site typically contains several polar and aromatic amino acids. The amino acids interacting within the −2 to +1 subsites of hemicellulose are highly conserved, with the catalytic network centered around two glutamic acid residues, which are critical for the enzyme's catalytic activity. These residues are considered catalytic due to their importance in the enzyme's function. Furthermore, three aromatic amino acids — Y77, W79, and Y171 — are located near the −2 subsites of hemicellulose and are believed to play a role in stabilizing the xylanose ring structure, which is essential for the formation of a bond with the substrate (Collins et al., 2005; Malhotra & Chapadgaonkar, 2018).

3.4.4. Lipases

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are enzymes that catalyze the hydrolysis of fats and oils, releasing free fatty acids, diglycerides, monoglycerides, and glycerol. In organic solvents, these enzymes also facilitate synthetic reactions such as esterification, acidolysis, alcoholysis, and interesterification. Lipases operate under mild conditions, exhibit high stability in organic solvents, and possess broad substrate specificity, often demonstrating high regio- and stereoselectivity in catalytic reactions. These features make lipases one of the most widely used biocatalysts in biotechnological applications (Moraleda-Muñoz & Shimkets, 2007). They are employed in various industries, including food, detergents, cosmetics, pharmaceuticals, leather, textiles, and paper, as well as in the production of biodiesel, biopolymers, and in the treatment of lipid-rich wastewater. These enzymes are found in a wide range of organisms, including animals, plants, bacteria, and fungi, with microbial lipases being particularly attractive due to their versatility and ease of large-scale production (Ergan et al., 1990; Karadzic et al., 2006)

Despite exhibiting low sequence identity in their primary structure, lipases share a similar structural fold. Other enzymes, such as esterases, proteases, dehalogenases, epoxide hydrolases, and peroxidases, display comparable structural features and together with lipases form the α/β-hydrolase family (Lan et al., 2015). The α/β-hydrolase fold consists of a central β-sheet composed of eight parallel β-strands, except for β2, which is antiparallel to the others. This

sheet adopts a left-handed superhelical twist, creating a 90° angle between the first and last strands. Strands β3 to β8 are connected by a bundle of helices, where helices A and F are positioned against the concave side of the central β-sheet, while helices B, C, D, and E pack against the convex side (Mead et al., 2002).

The active site of α/β-hydrolases contains a highly conserved catalytic triad composed of a nucleophilic residue (serine, cysteine, or aspartic acid), a catalytic acid residue (aspartic acid or glutamic acid), and a histidine residue. In lipases, the nucleophilic residue is always serine. The nucleophilic residue in lipases is found within a highly conserved pentapeptide sequence, Sm-X-Nu-X-Sm, where Sm represents a small residue, typically glycine, but occasionally substituted by alanine, valine, serine, or threonine; X represents any amino acid; and Nu corresponds to the nucleophilic residue. This pentapeptide forms a sharp γ-turn between the β5 strand and the αC helix, known as the "nucleophilic elbow". The conformation of this strand-loop-helix motif forces the nucleophilic residue into energetically unfavorable backbone dihedral angles, creating steric constraints on neighboring residues. The "nucleophilic elbow" is considered the most conserved structural feature of the α/β-hydrolase fold (Brocca et al., 2009; Romero et al., 2013).

3.4.5. Optimal conditions for enzyme production by *Aspergillus*

In the past decade, the production of amylases using fungi from the *Aspergillus* genus has gained significant attention due to its importance in industries such as food processing and biofuels. Recent studies have demonstrated that *Aspergillus oryzae* can produce α-amylase using low-cost substrates like edible oil cakes, highlighting its potential in sustainable industrial processes (Balakrishnan et al., 2021). Additionally, a starch-hydrolyzing α-amylase produced by *Aspergillus niger* has been characterized, showing high acid tolerance and efficiency in starch hydrolysis, making it suitable for applications in the food industry (S. Wang et al., 2016). Furthermore, optimizing fermentation conditions, such as temperature, pH, and substrate concentration, has significantly enhanced the production of amylases by *Aspergillus terreus*, using pearl millet as a substrate in solid-state fermentation (A. K. Kumar & Sharma, 2017). These findings underscore the importance of *Aspergillus* in efficient amylase production for various industrial applications. Some examples of amylases produced by *Aspergillus* are shown in Table 4.

The production of cellulases by *Aspergillus* species has been a focal point in biotechnological research due to its relevance in the conversion of lignocellulosic biomass into valuable products. Studies have highlighted the efficiency of *Aspergillus niger* in utilizing

agricultural residues, such as sugarcane bagasse, as substrates for cellulase production under submerged fermentation conditions (Bhardwaj et al., 2019). Environmental factors like pH have been identified as crucial in enhancing cellulase synthesis, with specific adjustments yielding significant increases in enzymatic activity. Innovations in fermentation strategies, such as employing biofilm-based methods, have shown remarkable improvements in enzyme productivity, with biofilm fermentation increasing cellulase yields by over 50% compared to conventional methods (Gamarra et al., 2010). Some examples of cellulases produced by *Aspergillus* are shown in Table 4.

Recently, various commercial sectors have explored xylanases in processes such as wood pulp biolixiviation, paper manufacturing, food and liquid production, animal nutrition, and bioethanol. Due to their biotechnological properties, xylanases are often produced by microorganisms for industrial applications. Nature is teeming with microorganisms that generate enzymatic complexes capable of degrading cellulose and releasing hemicellulose-derived sugars, which are used in the production of products at highly competitive costs (Belorkar & Gupta, 2016). Microbial xylanases play a critical role in industrial processes. Most commercial enzymes are derived from mesophilic microorganisms, while extremophilic microorganisms, which are capable of surviving and thriving in extreme environments, allow for the use of thermal strategies in the development of industrial processes. Organisms such as bacteria, fungi, and yeasts are known to produce xylanase in natural systems (Chakdar et al., 2016; Malhotra & Chapadgaonkar, 2018). Xylanase enzymes have gained significant popularity in recent decades due to their primary applications in the paper and pulp industry, animal feed processing, beverage clarification, and the production of biofuels from agricultural waste (Gruening de Mattos et al., 2024; Kaushal et al., 2021).

Table 4. Enzimes production by different strains of *Aspergillus.*

Enzyme	Microorganism	Main substrate	pH	Temperature (°C)	Time of incubation (days)	Enzymatic activity	Reference
α-amylase	*Aspergillus flavus* AUMC10636	Soluble starch	5	30	7	22.68 U/mL	(Beltagy et al., 2022)
	Aspergillus ochraceus	Starch	-	37	5	1415 U/mL	(Devi et al., 2022)
	Aspergillus tamarii MTCC5152	Wheat bran	6.7	28	4	519.40 U/gds	(Premalatha et al., 2023)
	Aspergillus flavus S2-OY	Potato peel	5	35	3	5 U/mL	(Olakusehin & Oyedeji, 2021)

	Aspergillus terreus	Pomegranate peel	6	30	5	340.69 U/ml	(Ahmed et al., 2020)
	Aspergillus oryzae	Groundnut oil cake	4.7	32.5	4.5	9868.12 U/gds	(Balakrishnan et al., 2021)
	Aspergillus oryzae	Soybean husk and flour mill	6	30	6	47,000 U/gds	(Melnichuk et al., 2020)
Glucoamylase	*Aspergillus wentii*	Starch	7	25	3	3.5 U/mL	(Lago et al., 2021)
	Aspergillus flavus	Wheat straw	5.5	30	12	13.89 U/gds	(Singhal et al., 2022)
Endoglucanase	*Aspergillus uvarum* CBS 121591	Carboxymethyl cellulose	7	37	3	2.706 U/mL	(Bhati et al., 2023)
	Aspergillus niger	*Arachis hypogaea* shells	4	40	5	87.69 U/mL	(Sulyman et al., 2020)
	Aspergillus fumigatus JCM 10253	Ragi husk	2	48.6	8	97.06 U/mL	(Saroj et al., 2021)
Beta-glucosidase	*Trichoderma reesei*	Cellulose	5	30	12	13.44 U/mL	(L. Shen et al., 2021)

3.5. New technologies and innovation of enzymes applied to biofuels

3.5.1. Protein engineering

Protein engineering encompasses a range of molecular and computational techniques aiming to modify the amino acid sequence in an enzyme to optimize its activity, expression, stability under different conditions, and substrate specificity (Yang et al., 2024). Traditional protein engineering relies on directed evolution or rational design. Directed evolution mimics Darwinian evolution at a higher mutation rate, with enzyme selection based on desirable properties. It involves two main steps: generating genetic diversity through random mutagenesis or gene recombination, followed by screening enzyme activity and selecting the best variants (Y. Wang et al., 2021). In contrast, enzyme engineering through rational design involves point mutations in the coding sequence, which requires prior knowledge of protein structure and function. In theory, rational design should be less labor intensive to perform than directed evolution; however, acquiring knowledge of the relationship between structure and function of the enzyme may require additional efforts if the information is not promptly available. Directed evolution and rational design can be merged into semi-rational design, in which structural information is used to select a promising region for generating genetic diversity, producing better targeted enzyme libraries (Z. Song et al., 2023). There have been numerous studies discussing

protein engineering of enzymes applied to biofuel production, with most of them focused on cellulases and lipases.

Cellulose is the main carbohydrate in lignocellulosic biomass and its depolymerization to glucose is a fundamental step to produce biofuels. This can be achieved by employing cellulases, which are enzymes tailored to hydrolyze glycosidic bonds in cellulose. Because of this, there is a great need to not only develop cellulases with higher and more specific activities, but also microbial platforms that express these enzymes in abundance. Researchers at the National Laboratory of Renewables (LNBR) at The National Center for Research on Energy and Materials (CNPEM), in Brazil, have developed a solution to this issue (Fonseca et al., 2020). The *Trichoderma reesei* RUT-C30 strain was engineered using CRISPR/Cas9 to produce the highest cellulase and xylanase levels ever reported, reaching 80.6 g/L of extracellular proteins. The enzyme cocktail showed saccharification efficiency comparable to commercial preparations used for sugarcane molasses. This hypersecreting strain was created by introducing recombinant invertase and β-glucosidase, constitutively expressing the cellulase regulator XYR1, and deleting the ACE1 repressor and extracellular proteases SLP1 and PEP1. Other studies have also focused on engineering *T. reesei* for overproduction of cellulase and xylanase, using techniques like RNAi-mediated gene silencing and inducer-free expression systems. For example, Arai et al. mutated the XYR1 regulator and expressed two cellulase regulators in *T. reesei* E1AB1, generating a strain that did not require inducers to overproduce cellulases and xylanases (Arai et al., 2022). Building a productive microbial platform for enzyme production is a strategy that has also been tested in *A. niger*. The antioxidant defense metabolism of *A. niger* was engineered to allow the overexpression of proteins. Extensive oxidative folding of proteins within the endoplasmic reticulum causes accelerated production of reactive oxygen species (ROS) in *A. niger*, which negatively affects the production of proteins in this species. By integrating different modules for ROS detoxification in *A. niger*, total protein production was augmented by 88%, also increasing the activity of glucoamylases (X. Chen et al., 2024).

The same molecular techniques employed to modify *T. reesei* have also been studied in *Aspergillus* sp. For instance, cellulase expression in *A. niger* and *A. nidulans* is under the control of the XIR1 regulator. Gene disruption in XIR1 regulator in these species by homologous recombination increased cellulase and xylanase activities. In another study, CRISPR/Cas9 technology was used to increase endoglucanase activity in *A. fumigatus* by 40%. This was achieved by integrating the eglA gene from *A. niger* into the conidial melanin *pksP* locus, resulting in recombinant albino colonies (Benites-Pariente et al., 2024). Zou G. *et al.* developed

a CRISPR/Cas9 ribonucleoprotein method to edit *T. reesei, Cordyceps militaris* and *A. oryzae*, overcoming the low efficiency of ribonucleoprotein transformation in these fungi (Zou et al., 2021). Another strategy to increase protein production in *Aspergillus sp.* is partly fusing enzyme coding sequences with overexpressed proteins coding sequences, so that both genetic codes are expressed at high levels (James et al., 2012; Le Gal-Coëffet et al., 1995). This demonstrates that not only *T. reesei*, but also *Aspergillus sp.*, has the potential to become an ideal microbial platform for lignocellulose deconstruction.

Xylanases have also been a target of protein engineering to enhance lignocellulose deconstruction for bioethanol production. Almost all native xylanases lose activity under industrial conditions, calling for modifications to increase their stability (Sürmeli & Şanlı-Mohamed, 2023). Thermal stability and pH stability of xylanase produced by *A. niger* were improved by site-directed mutagenesis. Initially, the amino acids to be modified were selected by visualizing the three-dimensional structure of the enzyme using a computational model. By substituting one glycine and one tyrosine with cysteine at positions 116 and 135, respectively, the engineering xylanase activity was preserved at 70 °C. These modifications also led to stability across a higher range of pH, from pH 4.5-6.0 to pH 5.0-7.0 (Q. Li et al., 2019). Molecular dynamics identified four highly flexible regions (HFR) of an acid-resistant xylanase from *A. niger* with potential to increase thermostability. Iterative saturation mutagenesis was used to modify these regions, resulting in eight mutants. By combining the mutations of HFR III and HFR IV, a thermostable variant was developed, retaining enzymatic activity at 80 °C and 90 °C, making it a robust candidate for bioethanol production (R. Li et al., 2024).

Lipase protein engineering to enhance expression and key properties is crucial for improving enzyme efficiency in biodiesel production. Several studies have focused on increasing lipase expression in *A. niger* by inserting strong promoters into the coding sequence. The glucoamylase PglaA promoter has been shown to induce high expression levels of eight different lipases in *A. niger* (Zhao et al., 2020). PgpdA is another strong promoter studied to increase lipase synthesis, with the advantage of enabling continuous expression without the need for inducer molecules (Kluge et al., 2018). Another strategy to increase lipase production is deleting genes for endogenous proteases. Deleting these genes allows for the overexpression of exogenous enzymes, as they are not cleaved after secretion by extracellular proteases. The deletion of aspartyl proteases (*PEPA* and *PEPB*) increased lipase and glucoamylase yield in one study (Mattern et al., 1992). Another study reported the reduction in protease expression by

overexpressing *amyR*, which acts as a repressor of the *PrtT* activator of protease synthesis in *A. niger* (Kamaruddin et al., 2018).

Biodiesel is produced by the transesterification reaction of fatty acids catalyzed by lipases. Triacylglycerols are the most common fatty acids utilized to produce biodiesel, with many advances in protein engineering to improve lipases that catalyze reactions with this substrate (Sandaka et al., 2022; Svendsen, n.d.; Tian et al., 2021). However, the engineering of mono- and diacylglycerol lipases (MDGLs) is a prominent field with an urgent need for developments once mono- and diacylglycerols are an underexplored source for biodiesel. Lan et al. characterized and modified an *A. oryzae* MDGL to serve as a model for engineering more efficient lipases targeting fatty acids other than triacylglycerols. The authors solved the crystal structure of the *A. oryzae* lipase and compared it to other lipase structures to identify the residue V269 as a catalytically important amino acid. By testing different residue substitutions at that position, it was found that aspartic acid residue increased enzyme activity by six-fold by increasing affinity for mono- and diacylglycerols (Lan et al., 2021).

Although protein engineering is a valuable and established method for optimizing enzyme structure-function relationships, it is a time-consuming and complex process, with an enormous fitness landscape difficult to navigate manually. In biofuel production, this challenge is intensified by the urgent need to develop efficient processes that can compete with fossil fuels and address climate change. A promising approach to accelerate the design-build-test (DBT) cycle in protein engineering is the integration of machine learning (ML) algorithms with automated laboratories (Yang et al., 2024). ML models can analyze structure-function relationships of proteins much faster than humans, identifying potential sequences for modification and even generating new sequences with desired properties. Meanwhile, an automated testing station can generate, and test enzyme variants based on ML analysis, providing rapid feedback to accelerate the DBT process. Researchers at the University of Wisconsin-Madison developed self-driving autonomous machines for protein landscape exploration (SAMPLE) to optimize thermostability of glycoside hydrolases (Rapp et al., 2024). The ML model was built with data on the enzyme structure and catalytic activity, generating a fitness landscape analyzed by a Gaussian process model, which captures patterns from limited experimental data, after 10.000 simulations, 83% of the active sequences were correctly annotated by the ML model, which then proposed optimized sequences using Bayesian optimization (Rapp et al., 2024). These sequences were sent to an automated robotic laboratory, where DNA fragments were constructed and amplified by PCR reactions. Genetic circuits for

expressing the designed genes were built using the Golden Gate methodology (Yang et al., 2024). The generated genetic codes were expressed in a cell-free system based on T7 polymerase, and the thermal denaturation of the synthesized enzymes was immediately tested. Each DBT cycle lasted around 9 hours, and all four engineered enzymes became more thermotolerant, increasing their denaturation temperature by at least 12 °C. This study demonstrates that ML models and automated laboratories can accelerate the protein engineering process, enabling faster development of optimized enzymes for biofuels production (Rapp et al., 2024).

3.5.2. Enzymatic Immobilization

Immobilization is a traditional method used to enhance enzyme reusability, recovery, and stability under harsh conditions. Xue et al. developed a bioprocess for integrated aerobic cellulase production from *A. niger* in a synthetic medium, followed by saccharification of NaOH-pretreated corn stover and anaerobic bioethanol fermentation using *S. cerevisiae* in the same gas lift bioreactor. *A. niger* grew and produced cellulases in a wire mesh cylinder inside the bioreactor. After 48 hours, oxygen was replaced by N2, and the fermentation broth was pumped into the reactor for bioethanol production by immobilized *S. cerevisiae*. Cellulase activity exceeded 6.28 U/mL over four consecutive batches, and ethanol yield reached 45.9 g/L after 48 hours (Xue et al., 2020). Directed evolution was used to produce *A. uvarum* cellulases with high activities, which were then immobilized on alginate beads to enhance reusability. Mutagenesis with ethyl methanesulfonate (EMS) at 12% v/v resulted in cellulases with 1.4-fold and 1.8-fold higher activities in solid-state and submerged fermentation, respectively. Maximum activity of immobilized cellulase was achieved with 2% w/v sodium alginate and 0.2 M calcium chloride at 60 °C and pH 7.0. The immobilized enzyme retained 18.5% of its activity after five batches, demonstrating its potential for bioethanol production (Bhati et al., 2023). Magnetic nanoparticles are a versatile support for enzyme immobilization, enabling control of particle adsorption with a magnetic field. Cellulase from *A. niger* and *T. reesei*, and xylanase from *T. longibrachiatum* were immobilized on chitosan magnetic nanoparticles to hydrolyze NaOH-pretreated coconut husks. Tween 80 was added as a surfactant to enhance enzyme stability and prevent denaturation. At 2% (w/v) of Tween 80, immobilized cellulase from *A. niger* produced 0.412 mg reducing sugar/mL, demonstrating that this immobilization method with surfactant addition is a promising strategy to produce fermentable sugar (Tiatira et al., 2022).

Immobilizing xylanases is a key strategy for creating efficient enzyme cocktails for lignocellulosic biomass conversion into biofuels. Calcium alginate is a traditional support for

enzyme immobilization, but Jian et al. used a 3-D printer to immobilize xylanase from *A. oryzae* in various particle shapes. The optimized conditions of 1% (w/v) sodium alginate, 2% (w/v) $CaCl_2$, and a 10-minute crosslinking time resulted in the highest xylanase concentration inside the particle and minimal mass transfer limitations. Nearly 60% of immobilized xylanase was recovered after 7 cycles. Immobilized enzymes produced almost 50% more reducing sugars from corn cob hydrolysis than free xylanase, highlighting the potential of this technology, especially for bioethanol production from corn residues (Jiang et al., 2020). A biomimetic magnetic nanoparticle was developed to immobilize xylanase from *A. niger*, expressed in *Pichia pastoris*, with excellent reusability and enhanced storage stability. The nanoparticles were synthesized by adding MamC to an iron solution, achieving 87% enzyme immobilization at optimized concentrations of glutaraldehyde and EDC. The immobilized enzymes retained activity after 8 cycles of magnetic recovery, demonstrating the potential of this approach as a scalable technology (Salem et al., 2021).

The integration of starch hydrolysis with immobilized α-amylase and bioethanol production has been widely reported. Recently, α-amylase from *A. flavus* was immobilized on different supports using physical adsorption, ionic bonding, entrapment in gel, and covalent bonding. Immobilization via covalent bonding with 2% glutaraldehyde resulted in the highest starch conversion to reducing sugars (92%) and amylase activity (2522.2 U/mL). The ethanol yield from fermentation of the covalently bound amylase hydrolysate was twice as high as that obtained with free amylase (Beltagy et al., 2022). A novel liquid phase-air phase system was developed to produce amylase and bioethanol from cassava starch hydrolysate. The system consisted of a bioreactor connected to a reservoir by a siphon. *A. awamori* cells were immobilized inside the bioreactor, and the culture broth was siphoned into it, submerging the cells. Once the broth reached a critical level, it was siphoned back to the reservoir, exposing the cells to air. Alternating periods of submerged fermentation and air exposure (12 hours submerged and 3 hours submerged and 6 hours air exposure for raw and cassava starch, respectively) resulted in maximal glucoamylase and α-amylase expression. Simultaneous production of amylases and bioethanol was achieved by immobilizing *S. cerevisiae* in the reservoir, with ethanol yields of 0.46 g/g of starch and 1.73 g/g of starch per hour (Roble et al., 2020).

Metal-organic frameworks (MOFs) are gaining attention as a promising support for enzyme technology due to their superior stability and catalytic activity compared to traditional supports. The immobilization of *A. niger* lipases in modified zeolitic imidazolate frameworks (ZIF-8), specifically *Macroporous* M-ZIF-8, has been investigated to enhance enzyme diffusion.

This modification improved enzyme activity, recovery after repeated batches, thermal stability, and reusability compared to ZIF-8. Additionally, a 7-fold smaller concentration of lipase immobilized in M-ZIF-8 achieved the same activity as lipase in ZIF-8 for biodiesel production. The larger pores in M-ZIF-8 allowed more fatty acid methyl esters (FAME) to migrate into the particle, leading to higher biodiesel yields. After 24 hours, M-ZIF-8 immobilized lipases produced 80% FAME, while ZIF-8 immobilized lipases reached a plateau at 65% FAME due to enzyme denaturation. The lipases in M-ZIF-8 were protected from denaturation by methanol and glycerol due to the particle's structure (Hu et al., 2021). Lipases can also be absorbed onto the surface of ZIF-8. Xia et al. studied the absorption of lipases from *A. oryzae* on ZIF-8, demonstrating that hydrogen bonds, electrostatic interactions, and van der Waals forces did not affect the enzyme's conformation. The immobilization enhanced lipase stability in 10% methanol solution and across a wide pH range. It also improved FAME yield (81.19%) in biodiesel production from used cooking oil and retained 68.46% of lipase activity after five consecutive transesterification reactions. This highlights the significant potential of immobilization to enhance biodiesel production processes using lipases from *Aspergillus* species (Xia et al., 2025).

3.6. World market of biofuels' enzymes

In recent years, the market for enzymes applied in biofuel production has experienced significant growth, driven by advancements in biotechnological processes and the increasing demand for sustainable energy solutions. This market has been shaped by technological innovations, cost reductions, and the optimization of enzymatic formulations tailored to the specific needs of the industry. A notable example is the Beta Renewables biofuel plant in Italy, which integrates patented enzymatic cocktails primarily composed of cellulases and hemicellulases, designed for its physicochemical pretreatment process. These cocktails, valued between $5 and $8 USD/mL, are specifically formulated to enhance the hydrolysis efficiency of wheat straw and other lignocellulosic substrates. Beta Renewables collaborates with enzyme producers to develop formulations that address substrate variability and improve yields. These enzymes contribute to an annual production of approximately 30 million liters of bioethanol, highlighting their significance in large-scale processes (Lozowski, 2012).

In Brazil, companies like Raízen rely heavily on enzyme suppliers to optimize bioethanol production from sugarcane. Enzymatic solutions, particularly cellulases and β- glucosidases, valued at $4 to $6 USD/mL, are employed to maximize cellulose breakdown in sugarcane bagasse. Raízen's investment in enzymatic technologies, combined with physical and chemical pretreatments, supports its annual capacity of 2.5 billion liters of ethanol. The company

collaborates with global enzyme manufacturers to ensure a consistent supply and foster innovation in enzymatic formulations, reducing hydrolysis time and enhancing conversion efficiency (Neto et al., 2018).

GranBio, another Brazilian leader, focuses on second-generation bioethanol production using lignocellulosic residues. Its plant in São Miguel dos Campos incorporates tailored enzymatic blends composed of cellulases, hemicellulases, and β-glucosidases, specifically formulated for the hydrolysis of sugarcane bagasse and straw, with costs ranging from $5 to $9 USD/mL. GranBio's collaboration with enzyme suppliers has enabled the development of high-performance cellulases and hemicellulases that operate effectively under the physicochemical conditions of its processes. These enzymes represent a significant portion of production costs but are critical for achieving an annual output of over 80 million liters of bioethanol (Dos Santos et al., 2016).

Amyris, a Brazilian company known for its innovations in biodiesel, employs lipases for the enzymatic transesterification of vegetable oils and microalgal lipids. Its patented enzymatic formulations, composed of lipases specifically designed for triglyceride conversion and valued between $7 and $10 USD/mL, are aimed at improving lipid conversion efficiency while reducing by-product generation. Through investments in enzyme optimization, Amyris has achieved a 10% reduction in biodiesel production costs over the past five years, demonstrating the economic impact of advanced enzymatic technologies in biofuel production (Gray et al., 2009).

Despite these advancements, the market for biofuel enzymes faces challenges related to production costs, scalability, and market penetration. Enzymes for biofuels account for a substantial portion of operational expenses, with costs ranging from $0.3 to $1 USD per liter of biofuel, depending on their specificity and activity. Companies must balance enzyme costs with the need for high conversion efficiencies and process stability. Furthermore, the global enzyme market is highly competitive, dominated by key players such as Novozymes, DuPont, and DSM, who are continuously developing customized solutions for different substrates and pretreatment conditions.The future growth of the enzyme market depends on continuous innovation to reduce production costs and enhance enzyme stability under industrial conditions. Strategies such as enzyme immobilization, genetic engineering of enzyme-producing microorganisms, and co-culture systems promise to improve profitability and efficiency, driving the standardization of sustainable energy.

3.6.1. Global market analysis of enzymes utilized in biofuel production

To comprehensively assess the global market and the growing interest in biofuel production as a sustainable alternative to fossil fuels, it is essential to perform a contemporary patent analysis during periods of peak research activity in this field. For this purpose, the Derwent Innovations Index® database was utilized, applying the following search algorithm: TS=(*ASPERGILLUS*) OR TS=(AMYLASE) OR TS=(XYLANASE) OR TS=(CELLULASE) OR TS=(LIPASE) OR TS=(BIODIESEL) OR TS=(BIOETHANOL) and Derwent Class Code (DC)=(D16) and International Patent Classification (IPC)=(C12P-007/06) AND (C12N-015/80).

This search yielded 913 documents. To refine the dataset, only documents categorized under "Energy Fuels," a keyword provided by the Derwent Innovations Index®, were selected, resulting in a subset of 498 documents. Subsequently, documents with up to eight years of antiquity, corresponding to a period of heightened biotechnological biofuel production, were chosen, reducing the dataset to 111 documents. These records were exported to MS Excel® for manual screening, where titles and abstracts were analyzed to identify patents relevant to the study.

The analysis revealed that the majority of patents for biofuel production via enzymatic hydrolysis were filed predominantly by China and India, accounting for 60.32% and 14.29% of the total, respectively. This dominance can be attributed to the unique socio-economic and environmental challenges faced by these countries. Despite having petroleum reserves, both nations struggle to meet their growing domestic energy demands, which are exacerbated by their expanding populations. Additionally, the large volumes of waste generated in these countries present an opportunity for conversion into biofuels. The pressing need for environmentally friendly and economically viable energy solutions has driven China and India to innovate and protect new technologies for biofuel production (Figure 3).

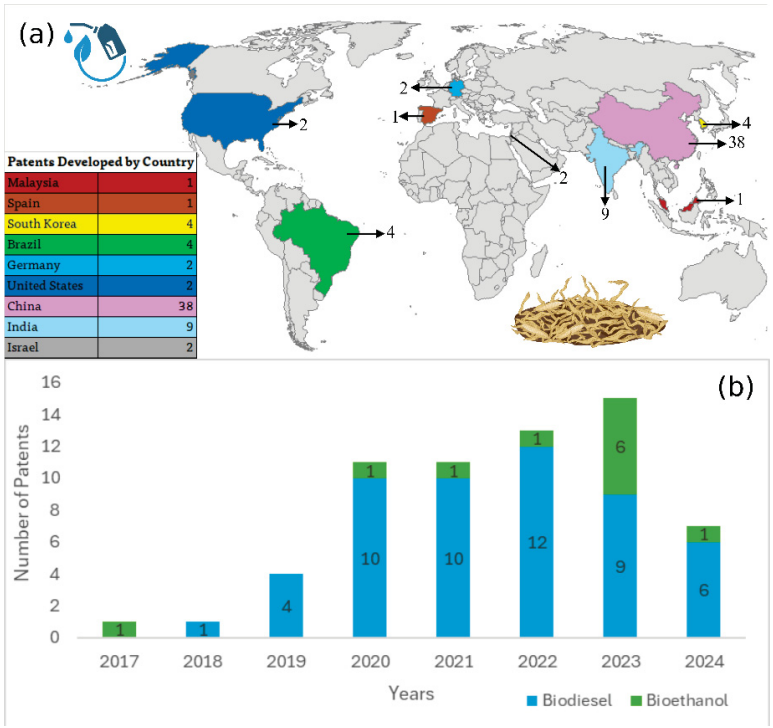

Figure 3: Global Patent Trends: (a)Distribution of Patent Filings by Country; (b) Timeline of Patents Filed Per Year.

The patents analyzed span an eight-year period, revealing a significant increase in the number of patents filed during 2020, 2021, 2022, and 2023, accounting for 17.46%, 17.46%, 20.63%, and 23.81% of the total, respectively (Figure 3). This trend can be attributed to the global impact of the COVID-19 pandemic, which exposed the critical dependency on fuel resources and highlighted the vulnerability of global supply chains in the face of resource depletion. As activities resumed in 2022, research efforts prioritized the development of new technologies aimed at addressing these challenges and strengthening the global biofuel market.

To facilitate a better classification and understanding of how these technologies are being developed, patents were grouped based on several factors: the type of biofuel produced, the raw materials utilized, the pre-treatments applied to these raw materials, the enzymes used for enzymatic hydrolysis or transesterification, the microorganisms responsible for enzyme production, and the institutions or companies protecting these innovations. All patents analyzed were associated with the "Waste valorization and biofuel production" application area, emphasizing a focus on developing a circular economy for viable biofuel production.

3.6.1.1. Biofuel types

The analysis highlighted that 79.37% of the patents filed focused on biodiesel production (Figure 4a). For instance, patent by Yang J & Zhang X, reported the immobilization of a fungal lipase from *Aspergillus sp*., used for the enzymatic transesterification of waste cooking oils pre-treated by centrifugation and decantation to remove contaminants. This technology, developed by Shanghai Zhongqi Environmental Protection Technology Co., Ltd. (Shanghai Shi, China, 2022), achieved a production yield of 98.2% (Yang J; & Zhang X, 2022). Another patent, by Xu G et al., focused on engineering a mutant lipase expressed in *E. coli* using genes from *Aspergillus*. This lipase, applied to pretreated vegetable oils, improved production yield from 85% to 99%. This technology was developed by Hunan Fulige Biotechnology Co., Ltd. (Hunan, China, 2021) (Xu G; et al., 2022).

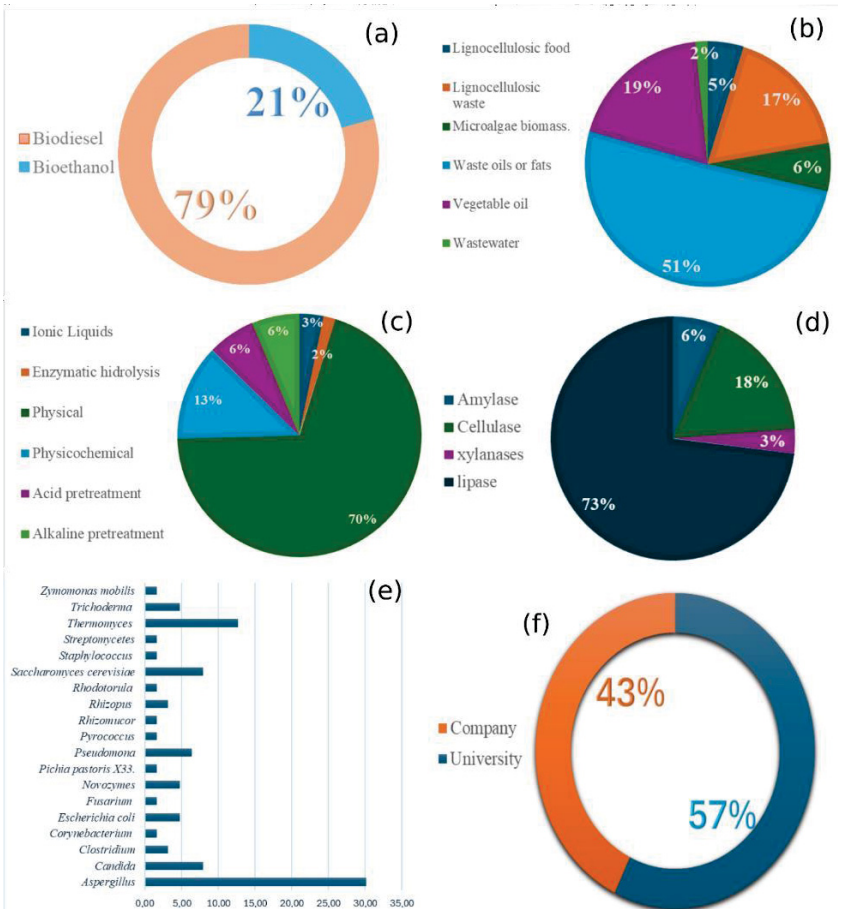

Figure 4. Statistical analysis of filed biofuel patents: (a): Distribution of biodiesel and bioethanol patents filed. (b): Raw material types used in biofuel production. (c): Pre-treatment methods applied to raw materials for biofuel production. (d): Enzymes utilized in biofuel production processes. (e):

Patents reporting microorganism utilization in biofuel production. (f): Key entities filing biofuel patents.

Only 20.63% of the patents analyzed addressed bioethanol production (Figure 4a), with limited data available. Of these, two patents utilized enzymes derived from *Aspergillus*. Patent of Yang T J et al. employed *Aspergillus sp*. to produce xylanases for enzymatic hydrolysis of agricultural residues pre-treated via milling and steam exposure to enhance surface area and partially remove lignin. This technology was developed by CJ CheilJedang Corporation (Seoul , South Korea, 2021) (Yang T J; et al., 2022). Patent of Singh A; & Pandey A K,, from Chhatrapati Shahu Ji Maharaj University (Uttar Pradesh, India, 2024), utilized *Aspergillus oryzae* to produce cellulases for enzymatic hydrolysis of sugarcane bagasse. Both patents relied on enzymatic hydrolysis to release fermentable sugars, which were later converted to bioethanol via fermentation with *Saccharomyces cerevisiae* (Singh A; & Pandey A K, 2024).

3.6.1.2. Raw materials and pre-treatment methods

Raw materials for biofuel production predominantly consisted of "waste oils or fats" (50.79%), followed by "vegetable oils" (19.05%) and "lignocellulosic waste" (17.46%) (Figure 4b). The pre-treatments applied were primarily physical and thermomechanical methods, including milling, decantation, centrifugation, cavitation, and autohydrolysis, which were reported in 69.84% of the patents analyzed (Figure 4c). A smaller proportion (12.70%) involved physicochemical pre-treatments, combining physical methods with hydrolysis or neutralization processes.

3.6.1.3. Enzymes and microorganisms

Enzymes used in biofuel production showed a marked preference for lipases, which accounted for 73.02% of patents, aligning with the dominance of biodiesel-related technologies (Figure 4d). In contrast, amylases, cellulases, and xylanases were collectively reported in only 26.98% of the patents. Microorganism analysis revealed that 30.16 % of the patents utilized enzymes produced by *Aspergillus*, while 12.70% used enzymes from *Thermomyces* (primarily for biodiesel production), and 7.94% involved enzymes from *Candida* (Figure. 4e). These findings suggest that *Aspergillus* remains underutilized for enzyme production in biofuel technologies, presenting opportunities for further research and optimization.

3.6.1.4. Institutions and companies filing patents

A detailed analysis of the entities filing these patents revealed that universities and research institutions were responsible for 57.14% of the filings, while companies accounted for

42.85% (Figure 4f). Notable contributors included Spanish National Research Council (CSIC), (Madrid, Spain), Perseo Biotechnology S.L., (Madrid, Spain), Shanghai Zhongqi Environmental Protection Technology Co., Ltd., (Shanghai, China), and Shaanxi Haisefu Biological Engineering Co., Ltd., (Xi'an, Shaanxi Province, China). While industrial interest in biofuel technologies is evident, these innovations still require significant optimization to reduce production costs and enhance accessibility for industrial markets.

These findings highlight the critical role of academia and industry in advancing biofuel technologies and the need for continued innovation to develop economically viable and environmentally sustainable alternatives to fossil fuels.

3.6.2. Relationship between feedstocks, pretreatments, and microorganisms in biofuel production

In response to the growing demand previously mentioned regarding the production of biofuels such as bioethanol and biodiesel, a chart was designed to highlight the various alternatives available to produce these high-energy-value compounds, with the primary objective of evaluating their viability and showcasing the synergy of technologies for industrial scaling. To analyze the current state of biofuel production from fungal enzymes from a bibliographic perspective, an exhaustive analysis of the sources cited in this scientific article was conducted to identify the key points in each investigation that led to favorable results. These references were represented in Figure 5. Following a network analysis, the influence and interrelations among their procedures were measured, in addition to identifying the main current trends in innovation for biofuel production.

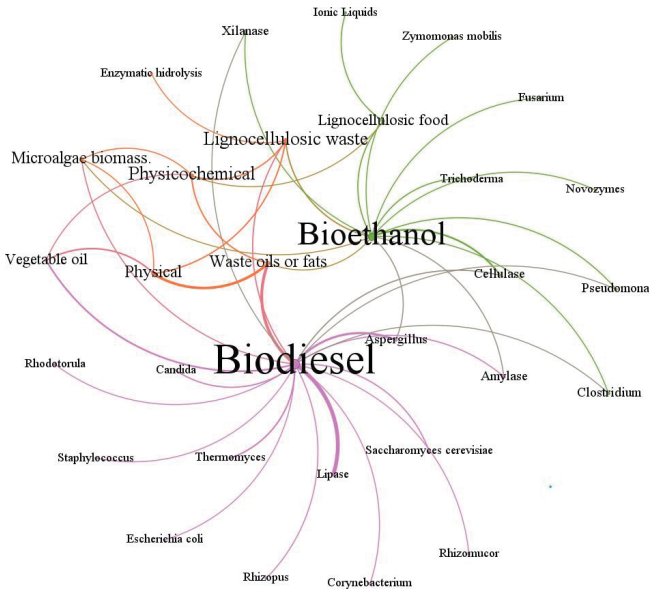

Figure 5. Network analysis of scientific terms used to identify emerging innovative trends in the production of bioethanol and biodiesel. Scientific landscape developed using the network analysis and visualization software, (Gephi 0.10.1).

The results highlight 'Bioethanol' and 'Biodiesel' as the most prominent biofuels produced from fungal metabolites, closely linked to microorganisms, organic waste, and production methodologies. The chart shows three main clusters: one for bioethanol (green), another for biodiesel (purple), and a third (orange) representing raw materials and pretreatments that connect the two main clusters. Smaller nodes reflect fewer common methodologies and microorganisms.

The primary challenge in bioethanol and biodiesel production lies in scaling technologies to industrial levels. Optimizing processes to valorize lignocellulosic biomass and residual oils could improve enzymatic complexes for waste treatment and biofuel production. However, this requires alignment with government regulations and institutional support. In Brazil, entities like IBAMA and SABESP oversee waste management and compliance with Law No. 12.305/2010, which promotes waste reduction, reuse, and recycling. Meanwhile, ANP, ANEEL, and APROBIO regulate biofuel production and energy generation from lignocellulosic waste, ensuring adherence to environmental standards and promoting sustainable practices.

According to performance studies, it has been established that the quality of the produced biofuels is closely linked to the type of pretreatment applied to the organic waste used (Alabdalall et al., 2023). As illustrated in Figure 5, generated from the published research, it has been shown that physicochemical pretreatments, which combine grinding processes with acid or alkaline hydrolysis, are among the most used methodologies today (Narendra Kumar et al., 2023). For example, Chugh et al. employed a grinding pretreatment followed by acid hydrolysis with 2% H_2SO_4 on rice bran, resulting in a significant increase in surface area, enabling a subsequent release of 468 mg of fermentable sugars per gram of hydrolyzed material using amylases and cellulases produced by the fungus *Aspergillus niger* P-19. These sugars were then fermented with *Saccharomyces cerevisiae*, producing 37.63 g/L or 0.41 g of bioethanol per gram of hydrolyzed material, which constitutes one of the best results obtained with this methodology (Chugh et al., 2023). On the other hand, despite the limited number of performance reports, the pretreatment of materials with ionic liquids, such as imidazole, presents itself as a promising alternative. As seen in Figure 5, this methodology appears somewhat more distanced from the main ones due to its limited bibliography, but still maintains a direct interaction with bioethanol production. Awodi et al., used ionic liquids to clean mango seed starch, followed by hydrolysis

of these substrates with α-amylase produced by *Aspergillus niger*, obtaining a concentration of 848 mg of fermentable sugars per gram of hydrolyzed substrate. This concentration was then subjected to a fermentation process with *Saccharomyces cerevisiae*, achieving a production of 31.40 g/L (Awodi et al., 2022). Comparatively, it can be stated that the treatment with ionic liquids effectively removed toxic materials for the enzymes and prevented the formation of inhibitors such as furfural, while preserving the chemical composition of the areas exposed to hydrolysis, keeping intact their properties for enzymatic release. The optimization of these technologies is crucial to identify the optimal point at which the number of applications of these liquids is maximized, allowing for the highest possible yield and turning this technology into a viable option for industrial scaling.

On the opposite side of Figure 5, the patents and studies analyzed and utilized in this research report the use of waste oils and animal fats. These materials, often discarded without undergoing recovery or neutralization pretreatments, represent a valuable resource for biodiesel production. Closely associated with physical pretreatments, these are the most predominant methodologies for biodiesel production, while physicochemical pretreatments are applied only when neutralization or denaturation of the waste material is necessary. For instance, Wei et al. demonstrated that the filtration of waste cooking oils is sufficient to remove impurities from this type of organic material. Using an immobilized lipase produced by the fungus *Aspergillus oryzae*, they achieved a 98.5% yield in the conversion of fatty acids to biodiesel through an enzymatic transesterification conducted at 40 °C for 9 hours (Wei et al., 2023). This approach represents one of the fastest methodologies reported for biodiesel production. In another case, Amoah et al. performed a natural sedimentation process followed by lyophilization of *Chlamydomonas sp*. JSC4 biomass. Subsequently, the intracellular lipids released were treated through lyophilization and lipases produced by *Aspergillus oryzae*, achieving a 97% yield in biodiesel production. However, this yield was obtained after 32 hours at 30 °C, which, while offering a favorable production temperature, limits industrial scaling due to the time required for production (Amoah et al., 2017). On the other hand, Singh A & Pandey A K, used plant oils, animal fats, waste oils, and microbial oils that were pretreated with acids until reaching a fatty acid concentration between 75% and 80%. These materials were utilized in the production of fatty acid methyl esters (FAME) using a lipase, which in this case was derived from the bacterium *Streptomycetes sp*. The enzymatic transesterification was carried out at temperatures of 40-60 °C for a duration of 6 to 10 hours at 200 rpm, employing a molar ratio of alcohol to biomass of 3:1 and low-carbon alcohols (C1-C4). This approach achieved a 93% yield, slightly lower than that reported for other enzymatic transesterifications. However, controlling the fatty

acid content in the raw material broadens the range of oil sources that can be used, reducing the quality requirements for the feedstock and laying the groundwork for process standardization(Singh A; & Pandey A K, 2024).

The fungus *Aspergillus* is a key microbial producer of industrial enzymes such as cellulases, xylanases, amylases, and lipases, widely used in bioethanol and biodiesel production. Studies show that a single *Aspergillus* strain can synthesize different enzymes depending on cultivation conditions. However, few patents for bioethanol production document the direct use of enzymes from this genus. Instead, the use of other microorganisms is reported, such as *Clostridium* (Jeon B H; et al., 2022), *Fusarium* (Kuila A; et al., 2022), *Zymomonas* (Dubey S, 2023), *Trichoderma* (Marathe A B; & Deshmukh M P, 2024) as well as the contribution of enzyme-supplying companies, such as Novozymes (Peinado CEBRIAN C; et al., 2024). This highlights the need for greater dissemination and optimization in the development of fungal enzymes to ensure their scalability and commercial viability. On the other hand, biodiesel production has shown a greater utilization of lipases produced by the fungus *Aspergillus*. Additionally, other lipase-producing microorganisms have been identified, such as *Rhodotorula* (ZhouHOU W; et al., 2023), *Staphylococcus* (Gao Q; et al., 2023), *Thermomyces* (Wang X; et al., 2024), *Rhizopus* (Meerabai R S; et al., 2021), *Corynebacterium* (Liu D; & Chen Z, 2024), *Rhizomucor* (Zhang J; et al., 2022), *Candida* (Wang J; et al., 2022) and *Saccharomyces cerevisiae* (Zhang H; & Chang J, 2024) the latter genetically modified to express optimized lipases. In conclusion, biofuel production demands continuous improvement in processes to ensure both sustainability and efficiency. This challenge is crucial not only to advance toward a cleaner energy matrix but also to preserve the necessary conditions to sustain life as we know it.

3.7. Conclusion

Fungal enzymes, particularly cellulases, xylanases, amylases, and lipases produced by the filamentous fungus *Aspergillus*, play a crucial role in the transformation of lignocellulosic biomass and oils into bioethanol and biodiesel, establishing themselves as key tools in the transition toward a more sustainable biofuel production. Recent advancements, such as the design of more stable enzymes using inorganic supports and the genetic engineering of *Aspergillus* strains, have enhanced the cellulose conversion efficiency by 30%. Additionally, the integration of hybrid pretreatments has reduced conversion times from weeks to days, achieving efficiencies above 90%. Noteworthy examples include the application of these fungi in conjunction with physicochemical technologies to enhance the release of fermentable sugars. Ionic liquids, despite their economic challenges, have shown potential to reduce costs by up to

30% by facilitating the dissolution of cellulose with high efficiency. Furthermore, recent studies have explored new agroindustrial substrates, such as mango peels, invasive plants, and starchy waste, where these enzymes have demonstrated their versatility in adapting to diverse chemical compositions, achieving bioethanol concentrations of up to 108.6 g/L from rice straw pretreated with alkaline methods. Moreover, the optimization of fermentation and transesterification processes using lipases has achieved biodiesel yields exceeding 97%, even with low-quality oils, underscoring the critical role of these enzymes in overcoming technical and economic barriers. In summary, *Aspergillus*-derived enzymes are not only essential for the valorization of lignocellulosic biomass and oils but also serve as the foundation for recent innovations that have improved efficiency and reduced costs in biofuel production. Future research should focus on customizing biocatalysts for extreme conditions, exploring new substrates, and integrating hybrid systems, aiming to build a more sustainable biofuel industry aligned with circular economy principles.

4. Optimization of the Production and Purification of β-Glucoamylase from *Aspergillus oryzae*

Development of an efficient process for production and partial purification of β-glucoamylase from *Aspergillus oryzae*

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Abstract

The production of enzymes in aqueous media is one of the most viable alternatives for the biofuel industry, especially amylolytic enzymes such as α-amylases and β-glucoamylases (GMA), which convert starch into glucose by catalyzing the alpha-1,4 and alpha-1,6 linkages in starch. This process is essential for glucose generation, which is used in fermentation for bioethanol production. This study aimed to optimize the production of β-glucoamylases through submerged fermentation (SmF) using milled corn in suspension, selecting *Aspergillus oryzae* LPB 1808 from 10 evaluated strains due to its initial enzymatic activity (14.29 U/mL). Statistical optimizations were performed using a fractional factorial design, Plackett-Burman design, and central composite rotatable design (DCCR), adjusting variables such as substrate concentration, pH, and medium composition. The optimal medium consisted of 5% milled corn, 2.06 g/L ammonium sulfate, 6 g/L dipotassium phosphate, 0.075 g/L calcium chloride, 0.25 g/L copper sulfate, 0.1 g/L magnesium sulfate, 0.05 g/L manganese sulfate, and 2 g/L meat extract, with an inoculum of 4×10^6 spores/mL at pH 4.5. Under these conditions, enzymatic activity reached 25.90 U/mL after three days of fermentation, with a productivity of 0.2158 U/mL·day. After concentration and pre-purification through micro and ultrafiltration, a final activity of 47.62 U/mL was achieved. SDS-PAGE analysis revealed a β-glucoamylase with a molecular weight of 60-65 kDa. The enzyme showed a glucose release of 0.232 g per gram of milled corn, comparable to commercial β-glucoamylase. Furthermore, the purified enzyme maintained a residual activity of 44.305 U/mL after 15 days of storage at 4°C, representing 93.03% of its initial activity. The results demostrate that β-glucoamylase production can be optimized using cost-effective substrates and controlled conditions, facilitating its application in industrial biofuel production. Additionally, the use of agro-industrial products represents and efficient strategy for the

valorization of agricultural by products, contributing to waste reduction and the development of more sustainable processes in the context of industrial biotechnology.

Keywords: β-glucoamylase, milled corn, *Aspergillus oryzae*, submerged fermentation, glucose.

4.1. Introduction

Corn (Zea mays L.) is the third most widely produced cereal globally, providing more than half of the caloric and protein needs by the population in developing countries. It is composed of approximately 60% starch, 8% protein, 3.7% essential oils, 15% water, 2% sugars, and 1.5% minerals (Y. Chen et al., 2024). It also contains fibers and vitamins in smaller quantities, as well as oleic and linoleic fatty acids, and carotenoids that affect the color of the corn (Fuentes-Cardenas et al., 2022). (Wu et al., 2024). Since 2000, technologies for its use in bioethanol production have been developed, currently producing 45 million cubic meters, which represents 60% of global production (Aghaei et al., 2022). On a dry weight basis, the average composition of milled corn is 9.47% protein, 2.70% lipids, 0.96% minerals, and 84.39% carbohydrates (Callegaro et al., 2005; Y. Chen et al., 2024; Giacomelli et al., 2012; Massarolo et al., 2019). Market studies estimate that by 2024, corn-derived products will reach 130.87 million tons, valued at USD 143.62 billion, with an annual growth projection of 3.01%, reaching USD 166.57 billion by 2029. Among these, milled corn stands out as a low-cost agro-industrial byproduct, traditionally underutilized, yet rich in starch, making it a promising raw material for biotechnological applications. Its use in the production of high-value fungal enzymes, such as β-glucoamylases, offers an opportunity to enhance its economic potential while contributing to more sustainable bioethanol production (Gumul et al., 2024; Harris et al., 2024).

β-glucoamylase (1,4-alpha-D-glucan glucohydrolase, EC 3.2.1.3) are digestive enzymes from the GH31 glycosidase family that, through Koshland's displacement mechanism, catalyze glucosylation and deglucosylation to change the configuration at the anomeric center. β-glucoamylase has two subunits with different specificities: the N-terminal domain has a higher affinity for maltose, maltotriose, maltotetraose, and maltopentose, while the C-terminal domain is broader, allowing the degradation of glucose oligomers. The production of β-glucoamylase has grown over the past century, with distribution in North America (50%), Europe (25%), Asia (10%), Africa (10%), and South America (5%). According to (Zaghetto de Almeida et al., 2024a), optimizing substrates for its production has improved product profitability and quality, making it a preferred choice for biorefinery processes. In 2018, the β-glucoamylase market reached USD 632.2 million and grew to USD 789.2 million in 2022, with an annual growth rate of 5.7%. It is expected that by 2033, the market will reach USD 1.283 billion.

According to Pazur et al., (1971), β-glucoamylases have a carbohydrate structural chain covalently bound to the enzyme, which includes D-mannose (67-128), D-glucose (16-20), D-galactose (2-3), and 2-amino-2-deoxy-D-glucose (0-20). This chain is linked to key amino acids that determine the enzyme's specificity and activity, such as aspartic acid (83-104), threonine (88-104), serine (108-121), glutamic acid (48-56), proline (29-31), glycine (59-77), alanine (78-87), cystine (6-8), valine (46-58), methionine (4-6), isoleucine (25-36), leucine (53-57), tyrosine (29-44), phenylalanine (27-34), lysine (16-39), histidine (6-8), arginine (18-23), and tryptophan (30-36). Additionally, ammonia molecules (21-53) may be present. Depending on its composition, β-glucoamylase has a molecular weight ranging from 49 kDa to 112 kDa. According to Y. Wang et al., (2024), active sites and metabolic pathways of β-glucoamylase have been extensively characterized, including specific domains such as a P9 trefoil domain with cysteine residues, an N-terminal beta-sandwich domain, a broad catalytic domain (beta/alpha) with two inserted loops, and similar domains in the C-terminal region. This C-terminal domain, which lacks sugar-binding sites +2/+3, is specific for maltose to maltopentaose molecules, while the C-terminal domain allows degradation of larger substrates through hydrolysis of 1-4 glycosidic bonds (Abdelwahab, 2020).

Organisms produce β-glucoamylases as part of their metabolism to degrade substrates and obtain energy. These enzymes, produced by animals, plants, and microorganisms, facilitate the hydrolysis of sugars and the release of chemical energy. Research on β-glucoamylase production began in the 20th century (Pandey, 1995), and various methods have been explored to optimize its production, including alternative microorganisms and more cost-effective fermentation methods. Bacteria, fungi, and yeasts have been used for enzyme production (Reihani et al., 2024; Zaghetto de Almeida et al., 2024a; Zong et al., 2024). Fungi are considered the best producers due to their intermediate metabolic pathways (R. Li et al., 2024). Fungal metabolite production occurs through two types of fermentation: solid-state fermentation (SSF) (Fadel et al., 2020) and submerged fermentation (SmF) (Benassi et al., 2014; Zaghetto de Almeida et al., 2024a), with the latter being preferred for its scalability (Gaddam, 2024; K. K. Valladares-Diestra, Porto de Souza Vandenberghe, & Soccol, 2021).

The main objective of this study was to evaluate and optimize the production of β-glucoamylase through submerged fermentation using the fungus *Aspergillus oryzae* NRRL1808. Initially, a kinetic study was conducted using a previously reported fermentation medium, followed by process condition optimization through statistical analysis with milled corn as a substrate at the laboratory scale. Finally, the enzyme was partially purified.

4.2. Materials and Methods

4.2.1. Preparation of the fermentation substrate

The substrate utilized in this study was milled corn, which underwent a preliminary treatment to ensure its suitability for β-glucoamylase production in a submerged fermentation (SmF) system. This process began with the selection of intact and impurity-free corn kernels, discarding those exhibiting signs of deterioration or contamination. The selected grains were subsequently milled using a hammer mill to achieve a particle size of 0.5 mm. The substrate was then homogenized in a rotary mixer at 25°C for 30 minutes, ensuring uniformity of the material. Finally, the moisture content was adjusted to 50%, optimizing its availability as a carbon source for fungal growth, this substrate was supplied by the COAMO cooperative (Campo Mourão - PR - Brazil, 1975) and is characterized by its high starch concentration (75%), making it ideal for enzymatic production. Additionally, it contains proteins (10%), lipids (3%), and ash (1.5%), as well as essential ions such as potassium (0.53%), phosphorus (0.31%), magnesium (0.1%), and calcium (0.03%), to ensure the reproducibility of the fermentation process, the substrate was stored under controlled temperature and humidity conditions, preventing contamination and premature degradation. Furthermore, its compositional stability was confirmed to guarantee compatibility with the fermentation medium. (Y. Chen et al., 2024; Massarolo et al., 2019; Mojović et al., 2006).

4.2.2. *Screening and inoculum preparation of β-glucoamylase-producing microorganisms for submerged fermentation*

Strains of *Aspergillus* from the DEBB strain bank were used: *Aspergillus oryzae* LPB1989, LPB2217, LPB5593, LPB1808, LPB3485, LPB2220, LPB5321, and *Aspergillus niger* LPBW12, LPBIZ9, and LPB28, sourced from the strain collection of the Postgraduate Program in Bioprocess Engineering and Biotechnology (PPEGBB) at the Federal University of Paraná (UFPR) in Curitiba, Brazil. The fungus was stored on PDA at 4°C and regularly renewed. To evaluate amylolytic capacity, the Lugol test was used (S. Das et al., 2024). Ten fungal strains were inoculated in a modified peptone broth, with peptone (4 g/L), yeast extract (3 g/L), malt extract (3 g/L), and starch (10 g/L) as the main carbon source. The inoculum consisted of a solution with 4×10^6 spores/mL (K. K. Valladares-Diestra, Porto de Souza Vandenberghe, & Soccol, 2021). The production of amylolytic enzymes was measured over five days. The selected strain was cultivated on PDA, releasing its spores with 1% Tween 80, and quantified in a Neubauer chamber to reach the optimal concentration for efficient β-glucoamylase production.

4.2.2. *Preliminary tests and optimization of nitrogen sources and medium composition for β-glucoamylase production via submerged fermentation: screening of significant factors using central composite rotational design (CCRD).*

The β-glucoamylase production process began with submerged fermentation using a medium adapted from Gomes et al., (2005). The medium contained 1.0% carbon source (Soluble starch), 0.14% ammonium sulfate $(NH_4)_2SO_4$, 0.60% dipotassium phosphate (K_2HPO_4), 0.20% monopotassium phosphate (KH_2PO_4), 0.01% magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.50% yeast extract, 0.20% peptone, and 0.20% beef extract, with a pH of 5.0. The inoculum concentration was 4×10^6 spores/mL in 250 mL Erlenmeyer flasks, incubated at 28°C at 100 rpm for 2 days.

The optimization of the nitrogen source was carried out using a one-variable-at-a-time (OVAT) experimental design, without combining ammonium sulfate (21%), yeast extract (10.70%), bacterial peptone (12.5%), and urea (46.65%). The values in parentheses indicate the nitrogen content percentage for each source, and these were used to calculate the necessary amounts to match the nitrogen availability of the initial medium (Table 1). By including an initial point with the concentrations of the initial medium, it was possible to evaluate the individual impact of each source on the process.

Table 1: Optimization of nitrogen source for β-glucoamylase production

Substrate/Exp (150mL)	**1**	**3**	**4**	**5**	**6**
Yeast extract	0.75 g	1.1413 g	-	-	-
Bacterial peptone	0.3 g	-	0.97097g	-	-
Ammonium sulfate	0.21 g	-	-	0.62 g	-
Urea	-	-	-	-	0.2828 g

A Plackett-Burman (PB) experimental design screened significant factors (meat extract, $MgSO_4$, KH_2PO_4, K_2HPO_4, $FeSO_4$, $MnSO_4$, $ZnSO_4$, $CaCl_2$, and $CuSO_4$) affecting production, Table 2.

Table 2: Study of the influence of medium factors on β-glucoamylase production.

Factors	Levels (g/L)	
	0	1
Meat Extract	0	2
Magnesium Sulfate	0	0.1
Monobasic Potassium Phosphate	0	2
Dibasic Potassium Phosphate	0	6
Iron Sulfate	0	1
Manganese Sulfate	0	0.05
Zinc Sulfate	0	0.2
Calcium Chloride	0	0.05
Copper Sulfate	0	0.25

Followed by a fractionated factorial design incorporating positive components, resulting in a mathematical model for enzymatic activity (Table 3), with the first modification of the carbon source, replacing soluble starch with milled corn, an agro-industrial material produced after the grinding of dry corn kernels during the separation of starch from other components of interest through dry milling. (Y. Chen et al., 2024; Massarolo et al., 2019; Mojović et al., 2006).

Table 3: Influence of factors concentrations on β-glucoamylase production fractionated factorial design

Factors	Levels(g/L)		
	-1	0	1
Milled corn (carbon source)	5	10	15
Ammonium Sulfate $(NH_4)_2SO_4$	2.06	4.13	6.195
Dibasic Potassium Phosphate (K_2HPO_4)	3	6	9
Calcium Chloride ($CaCl_2$)	0.025	0.05	0.075
Copper Sulfate ($CuSO_4$)	0.125	0.25	0.375

Finally, the optimal concentrations of the previously identified compounds, were refined using Central Composite Rotational Design (CCRD) to enhance β-glucoamylase production through response surface methodology, Table 4.

Table 4: Coded and uncoded values of the factors studied in the CCRD for β-glucoamylase production optimization

Factors (g/L)	**Levels (g/L)**				
	-1.68	-1	0	+1	+1.68
Milled corn (Carbon source)	3.3	5	7.5	10	11.7
Ammonium Sulfate $(NH_4)_2SO_4$	1.3562	2.06	3.095	4.13	4.8338
Dibasic Potassium Phosphate (K_2HPO_4)	4.98	6	7.5	9	10.02

4.2.3. *Kinetics study of β-glucoamylase production in erlenmeyer flasks and stirred-tank reactor (STR)*

After determining the optimal fermentation medium concentrations, the kinetics of β-glucoamylase production were studied using 250 mL Erlenmeyer flasks, followed by fermenter stirred-tank reactor (STR) (Valladares-Diestra, K.K. et al.2021). The optimized medium, composed of the previously determined concentrations, was sterilized in an autoclave at 121°C for 15 minutes. After cooling and homogenizing the medium, the flasks were incubated at 120 rpm and 30°C for 120 hours, with sampling every 24 hours. All experiments were performed in triplicate. β-glucoamylase activity was determined by measuring the concentration of reducing sugars using the DNS (dinitrosalicylic acid) method.

Mass enzyme production was carried out in a MDL-750-10L fermenter, brand B.E. Marubishi (1327), with a working volume of 4 L, following the optimized conditions. The fermentation was conducted at 30°C, 300 rpm, and an aeration rate of 1.0 vvm. Samples were taken every 24 hours, and silicone oil (Dow Chemical Inc., USA) was added to prevent foam formation.

4.2.4. *Recovery, separation, and purification of β-glucoamylase using microfiltration, ultrafiltration, and SDS-PAGE analysis*

After submerged fermentation, the enzymatic extract was recovered and filtered using Whatman No. 1 filter paper with a vacuum-assisted Kitasato flask. The filtered extract was subjected to microfiltration and ultrafiltration using a Vivaflow® 200 system (Sartorius) with polyethersulfone (PES) membranes, with molecular weight cut-offs ranging from 30 to 100 kDa (Mendonça et al., 2023; Valladares-Diestra et al. 2021). The retentate and permeate fractions were recovered for analysis of enzymatic activity, protein concentration, and specific activity (Zaghetto de Almeida et al., 2024a). The filtered samples were diluted with a loading buffer in a 3:1 ratio and subjected to 10% SDS-PAGE electrophoresis (Pasin et al., 2017; Y. Wang et al., 2024), using a Wide Range molecular weight marker (200 kDa–6.5 kDa) from Sigma-Aldrich, gradually increasing the voltage from 60 V to 110 V over 2 hours (Zaghetto de Almeida et al., 2024). The gel was fixed with a solution of 50% methanol, 10% acetic acid, and 0.77% (w/v) ammonium acetate, and stained with Coomassie Blue for one day. Finally, the gel was cleaned with a 20% acetic acid solution for 30 minutes, and the molecular bands were visualized under UV light, allowing for clear observation of the enzyme presence (Pasin et al., 2017).

4.2.5. *Characterization of β-glucoamylase and definition of optimal parameters for enzymatic activity*

The study of enzymatic parameters focused on the influence of pH and temperature on enzymatic activity using two experimental designs. The first was a One Factor At a Time (OFAT) design, where, based on reports from studies with fungal glucoamylases, a temperature of 55°C and a pH of 5 were set (Zaghetto de Almeida et al., 2024). The second design was a central composite rotational design (CCRD) with Design Expert software, which enabled the generation of response surface graphs. The experimental design included two factors: temperature (47.9 to 61.0 ºC) and pH (3.6 to 6.4), with five different levels, resulting in a total of 11 experiments, including 3 central points. With the optimal parameters found, enzymatic kinetics tests were performed.

4.2.6. *Assessment of Enzymatic Activity and Protein Concentration*

The enzymatic activity study was conducted using the methodology of (Zaghetto de Almeida et al., (2024), which involves reacting 500 µL of enzymatic extract with 500 µL of starch in a 0.1 M sodium acetate buffer (pH 4.5) at 55°C for 10 minutes. The reaction was then stopped with 125 µL of DNS reagent and heated. The glucose concentration was measured by spectrophotometry at 540 nm using a BIOTEK PowerWave XS spectrophotometer. Amylolytic

activity was determined by evaluating the total release of reducing sugars according to the Miller, (1959) method. One unit of enzymatic activity (U) is defined as the release of μmol of product per minute under the assay conditions. The results were expressed as U/mL for enzymatic activity and U/mg for specific activity (Bagheri et al., 2014; Zaghetto de Almeida et al., 2024). The protein concentration was measured using the Bradford, (1976) method, by mixing 150 μL of the enzymatic extract with Bradford reagent and reading absorbance at 595 nm.

4.2.7. *Formulation of the β-glucoamylase enzymatic extract.*

The enzymatic extract obtained from ultrafiltration was used to formulate an enzyme complex, following the study by Silva, (2017). Solution A and solution B were used as stabilizing additives. The residual activity of β-glucoamylase was evaluated at 5, 10, and 15 days of storage at room temperature, 4°C, 55°C, and 20°C (ambient temperature according to Weather Spark). The additives A and B, at concentrations of 1% and 5%, respectively, are approved for food applications.

4.2.8. *Application of β-glucoamylase for the release of total reducing sugars from agro-industrial product (Milled corn).*

To evaluate the activity of the produced β-glucoamylase, an agro-industrial product (milled corn) was used as a substrate in a hydrolysis process. First, was hydrolyzed with an alpha-amylase in a 0.02M phosphate buffer, pH 7, at 85°C for 2 hours to degrade the starch and release maltose molecules. The supernatant was filtered to remove the suspended solids from the milled corn and was hydrolyzed in parallel with a commercial β-glucoamylase and the produced fungal enzyme. Both commercial enzymes were donated by Usina Ouro Verde; no specific codes are available for these enzymes. The commercial β-glucoamylase, with an activity of 11,414.69 U/mL, was diluted 250 times to achieve an activity of 45.65 U/mL, similar to that of the fungal enzyme (47.625 U/mL). The reaction was carried out under the previously determined optimal conditions (53.87°C). The capacity to generate glucose was evaluated at 24 and 48 hours using HPLC (Arora et al., 2017; Pasin et al., 2024).

4.3. Results and discussion

4.3.1. *Screening and inoculum preparation of β-glucoamylase -producing microorganisms for submerged fermentation.*

The strain *Aspergillus oryzae* LPB1808 was identified as the most promising for β-glucoamylase production among 10 evaluated strains. Figure 1(A) shows the morphological characteristics of its colonies, including mature green spores and a cotton-like surface

appearance in PDA medium. Amylolytic activity was confirmed using the Lugol test, which revealed a brown coloration indicating starch degradation in the culture medium, as shown in Figure 1(B) (S. Das et al., 2024). Figure 1(C) provides details of the microscopic morphology of the studied fungus.

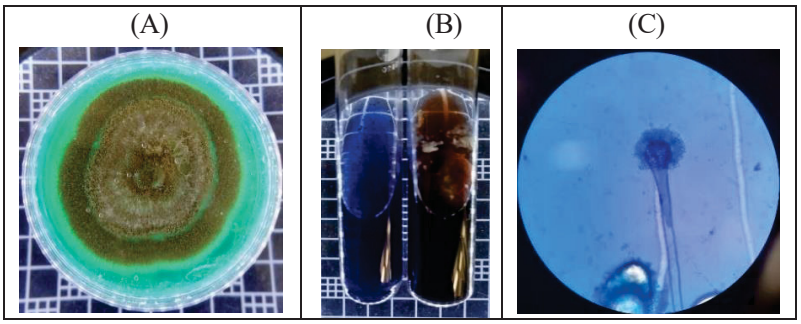

Fig. 1: Identification of the filamentous fungus: (A) morphology on plate, (B) qualitative analysis of starch degradation, (C) microscopy of fungal organelles.

Aspergillus oryzae LPB1808 demonstrated the ability to produce a β-glucoamylase with superior enzymatic activity. Figure 2 presents the kinetic profiles of β-glucoamylase production for each of the strains evaluated in a fermentation medium previously reported for β-glucoamylase production, exhibiting a higher activity (14.29 U/mL) compared to the other species. The production kinetics were monitored over a period of seven days, which was necessary for fungal strain growth and maturation. These results justify the selection of *Aspergillus oryzae* LPB1808 for the optimization and scale-up phases.

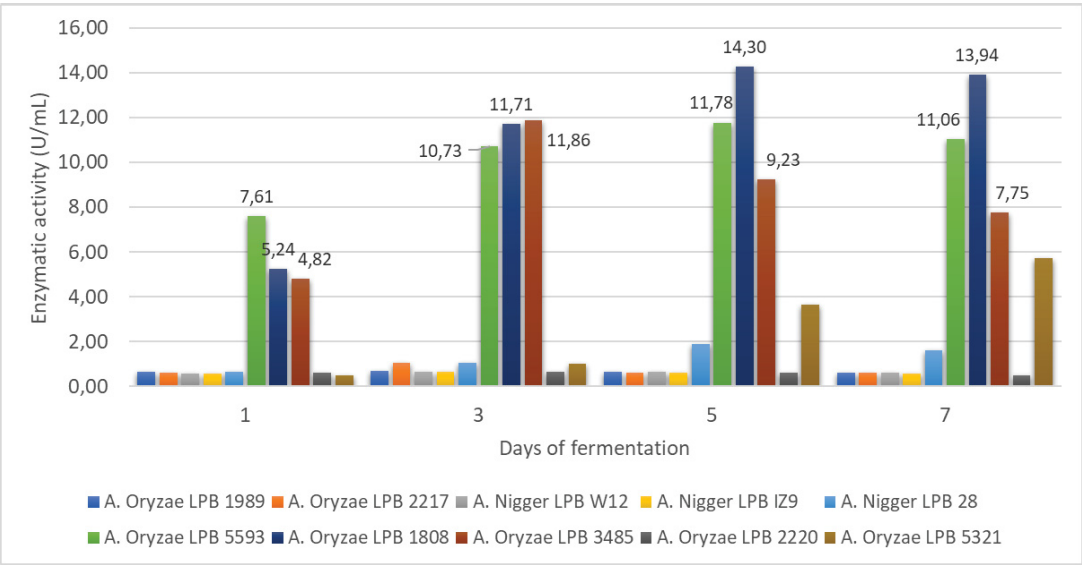

Fig. 2: Enzymatic activity over fermentation days.

4.3.2. *Preliminary tests and optimization of nitrogen sources and medium composition for β-glucoamylase production via submerged fermentation: screening of significant factors using central composite rotational design (CCRD).*

The fungus *Aspergillus oryzae* LPB1808 exhibited higher initial enzymatic activity (14.29 U/mL) in an unoptimized initial culture medium. In a preliminary optimization (Fig. 3), the medium containing ammonium sulfate showed similar activity (13.75 U/mL) to the initial medium and stood out for its low cost and availability, reducing production costs. When commercial starch (1% w/v) was replaced with milled corn (1% w/v), enzymatic activity decreased to 8.87 U/mL, attributed to the lower starch content in milled corn (75%) and the presence of secondary compounds that restricted enzymatic production.

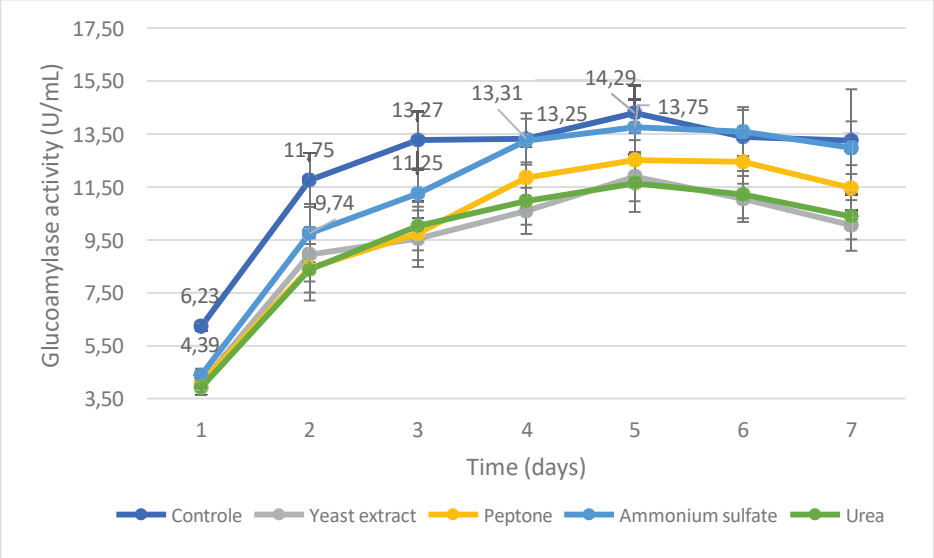

Fig. 3: β-glucoamylase activity produced with different nitrogen sources

From the Plackett-Burman (PB) design, a Pareto chart (Fig. 4) was generated to evaluate the impact of secondary compounds on the enzymatic activity of the β-glucoamylase produced. It was confirmed that dipotassium phosphate, calcium chloride, and, to a lesser extent, copper sulfate significantly improved β-glucoamylase production ($p < 0.05$), acting as essential cofactors or activators contributing to cellular nutrition and osmotic balance (Mendonça et al., 2023). The presence of dipotassium phosphate and calcium chloride was crucial for enzyme production, as reported by Gaddam, (2024); Pérez-Rodríguez et al., (2023); Reihani et al., (2024). Other compounds, such as manganese sulfate, magnesium sulfate, monobasic potassium phosphate, and beef extract, also enhanced enzymatic activity (Mendonça et al., 2023). In contrast, zinc sulfate, monobasic potassium phosphate, and iron sulfate were excluded from the fermentation medium due to their negative impact. ANOVA analysis in STATISTICA V.10

supported these conclusions, showing an R-squared value of 0.98866 and an MS Residual of 0.5864692.

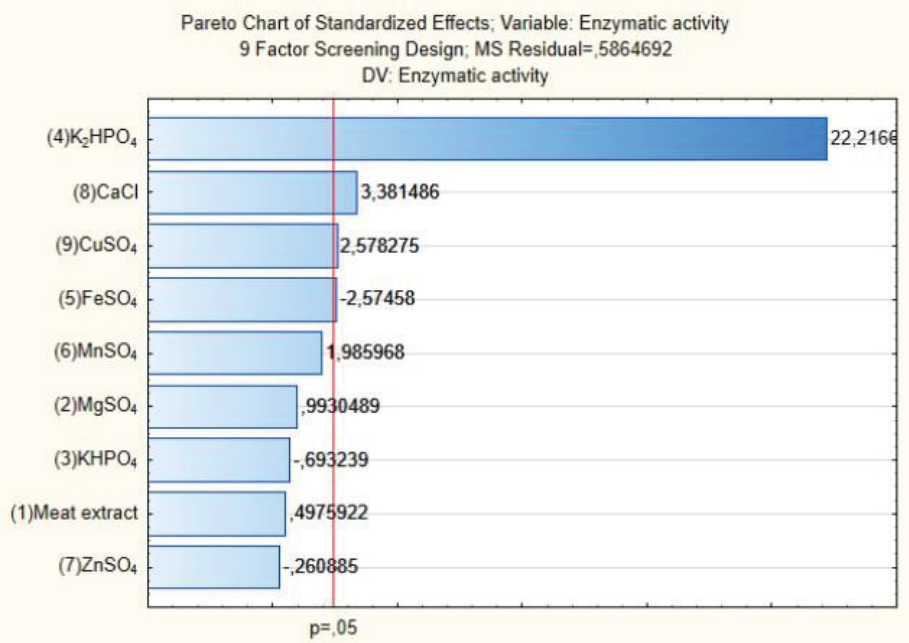

Fig. 4: Pareto chart showing the effect of different salts on β-glucoamylase activity

In a fractionated factorial design, the most impactful factors from the previous design were evaluated: dipotassium phosphate, calcium chloride, and copper sulfate. The compounds with positive effects were maintained at the previously analyzed concentrations. In this design, the key factors for β-glucoamylase production were also included: milled corn (carbon source), ammonium sulfate (nitrogen source), dipotassium phosphate, calcium chloride, and copper sulfate. It was observed that high concentrations of milled corn negatively affect enzymatic production, while the concentration of dipotassium phosphate has a positive effect and could improve results if increased in future designs. However, the ANOVA analysis conducted in STATISTICA V.10 showed an R-squared of 0.25866 and a Residual Mean Square (MS Residual) of 11.5942, making the design insignificant and highlighting the independent behavior of the compounds in β-glucoamylase production. The main purpose was to reduce factors for the central composite rotational design (CCRD). Dipotassium phosphate emerged as a key compound. The experiments from the CCRD design, explained in Table 4, showed variations in β-glucoamylase activity, ranging from 8.27 to 18.286 U/mL, as expressed in Table 5. Compared to the initial activity of 14.29 U/mL, this represents a maximum increase of 28%, although some conditions resulted in lower activity. The model was validated with an R^2 of 0.8701, $p < 0.02$, and an F value of 5.21, confirming its relevance and significant interactions. Significant terms such as the concentration of milled corn (A), ammonium sulfate (B), and dipotassium phosphate

(C), as well as their interactions AC, AB, BC, A^2, B^2, and C^2, were considered for the formulation of Equation 1.

Table 5: Resolved CCRD experimental design.

Run	Milled corn	(NH4)2SO4	2HPO4	Enzymatic activity (U/mL)
1	0	0	1.68179	11.2564
2	1	1	1	11.1485
3	-1	1	-1	14.1253
4	1.68179	0	0	6.0013
5	1	1	1	11.0908
6	0	0	-1.68179	16.6356
7	-1	1	1	12.2451
8	0	1.68179	0	8.2771
9	0	0	0	16.7284
10	0	-1.68179	0	16.8447
11	-1.68179	0	0	17.4715
12	0	0	0	16.1873
13	1	-1	-1	14.3253
14	-1	-1	-1	18.8286
15	1	-1	1	13.0125
16	0	0	0	16.2149
17	-1	-1	1	14.3322

$$Glucoamylase\ activity\ (\frac{U}{mL}) = 16.30527 - 2.14139 * A - 1.92561 * B - 1.22970 * C + 0.211487 * A * B + 0.625762 * A * C + 0.483912 * B * C - 1.39402 * (A^2) - 1.10252 * (B^2) - 0.612813 * (C^2). (Equation\ 1)$$

The response surface graphs (Fig. 5) analyzed the interactions between key factors in β-glucoamylase production. Fig. 5 (A) evaluated the interaction between ammonium sulfate and dibasic potassium phosphate, keeping milled corn at a level of -1. Fig. 5 (B) examined the effect between milled corn and ammonium sulfate with dibasic potassium phosphate at a level of -1, and Fig. 5 (C) measured the interaction between dibasic potassium phosphate and milled corn, keeping ammonium sulfate at a level of -1. The response surface statistical analyses determined

the optimal levels for the fermentation medium: 5 g/L of milled corn, 2.06 g/L of $(NH_4)_2SO_4$, 6 g/L of K_2HPO_4, 0.075 g/L of $CaCl_2$, 0.25 g/L of $CuSO_4$, 0.1 g/L of $MgSO_4$, 0.2 g/L of meat extract, and 0.05 g/L of $MnSO_4$. The produced β-glucoamylase experimentally demonstrated an activity of 18.82 +/- 0.18 U/mL (3765.72 U/g of milled corn).

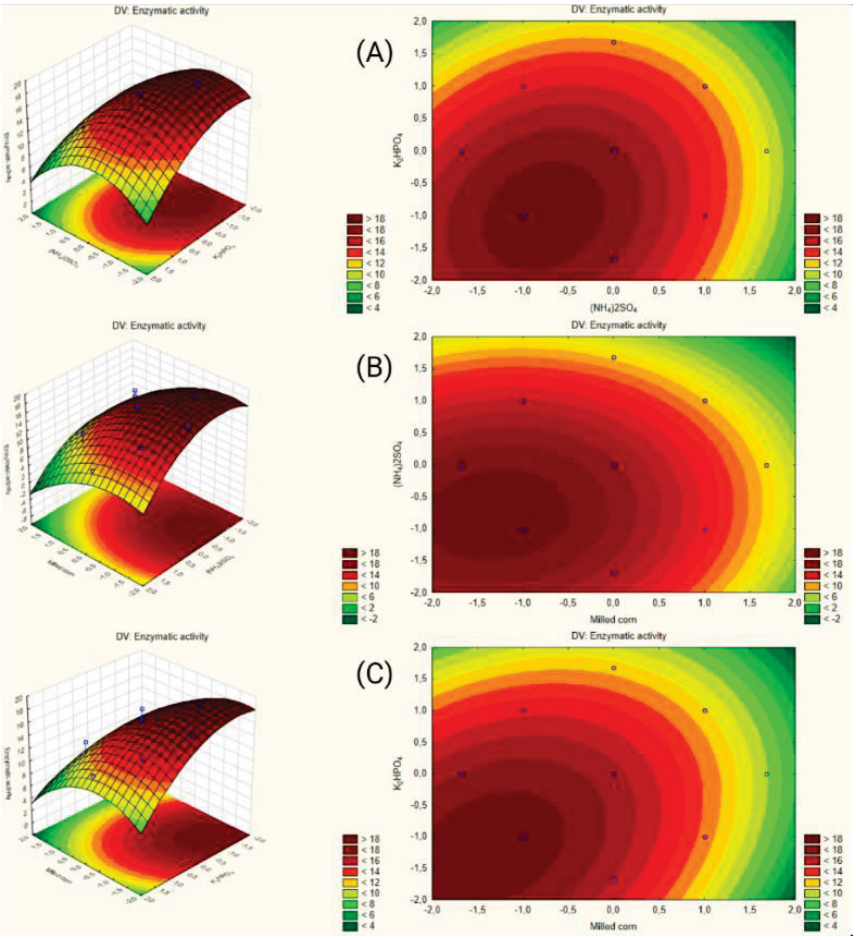

Fig.5: Three-dimensional surface response plot of the CCDR design and contour plot of the calculated response in the studied factors and their optimal levels, showing their influence on β-glucoamylase production.

4.3.3. *Kinetics study of β-glucoamylase production in erlenmeyer flasks and stirred-tank reactor (STR).*

After defining an optimal fermentation medium, a further study of the production kinetics was conducted, initially in Erlenmeyer flasks, with a comparison to the initial medium and the replacement of the carbon source at its initial concentration. As shown in Fig. 6, an increase in β-glucoamylase activity was observed compared to the previous results. The correlation between the experimental and statistical results validates the effectiveness of the response models and

confirms the existence of an optimal point for β-glucoamylase production. The highest recorded β-glucoamylase activity was 27.20 U/mL (0.1888 U/mL*h of productivity), achieved after 6 days of fermentation. However, the highest productivity was recorded on the third day (0.355 U/mL·day), despite a slightly lower production (25.60 U/mL).. Additionally, reducing the fermentation process by one day makes the process more economically viable.

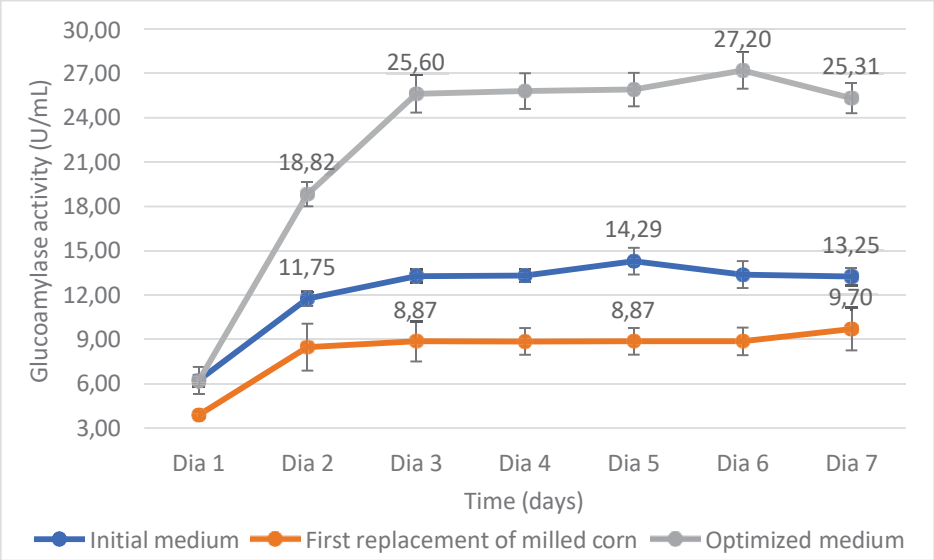

Fig. 6: Time course of β-glucoamylase production by *Aspergillus oryzae* NRRL 1808 After comparing the data obtained with the fungus *Aspergillus oryzae* LPB1808 with those reported in the literature, it was observed that the enzyme produced exhibits higher enzymatic activity compared to other fungi reported in the literature, such as *Aspergillus wentii* PG18 (3.5 U/mL) (Lago et al., 2021), *Aspergillus oryzae* LZ2 (11.58 U/mL) (C. Wang et al., 2020), and *Aspergillus oryzae* F-923 (8.56 U/mL) (Fadel et al., 2020), among others listed in Table 6, reaching 27.20 U/mL after 6 days of fermentation. It is only surpassed by the β-glucoamylase produced by the genetically modified fungus *Aspergillus niger* van Tieghem IBRC-M 30064 (33 U/mL) (Reihani et al., 2024), highlighting the impact of supplementation with Mg^{+2}, K^{+}, Ca^{+2}, and Cu^{+} ions on enzymatic production (Mendonça et al., 2023).

Table 6. Reports on β-glucoamylase production by different *Aspergillus* strains using various carbon sources

Microorganism	Carbon source	Concentration (g/L)	Product (U/mL)	Inoculum rate (spores/mL)	Productivity (U/mL*h)	Size	Ref.
***Aspergillus wentii* PG18**	Starch	2	3.5	$1*10^7$	0.0486	86KDa	(Lago et al., 2021)
***Aspergillus oryzae* LZ2**	Starch	10	11.58	$1*10^7$	0.120	60KDa	(C. Wang et al., 2020)
***Aspergillus clavatus* UEM 04**	Starch	2	4.5	$5.5*10^6$	0.0625	84KDa	(Mendonça et al., 2023)
Aspergillus niger	Fructose	10	0.6932	$5*10^7$	0.00577	60KDa	(K. Gupta & Nagar, 2020)
***Aspergillus oryzae* F-923**	Wheat bran Strach	50 50	8.56	$1*10^6$	0.118	65KDa	(Fadel et al., 2020)
***Aspergillus niger* van Tieghem IBRC-M 30064**	Corn starch Safflower meal	100 50	33	$1*10^6$	0.268	70KDa	(Reihani et al., 2024)
***Aspergillus brasiliensis* CFF124**	Maltose	10	7	$1*10^6$	0.0729	69KDa	(Zaghetto de Almeida et al., 2024a)
***Aspergillus oryzae* NRRL1808**	Millled corn	5	27.20	$4*10^6$	0.1888	60-65 KDa	This work

The productivity of the fungus was 0.2158 U/mLh, higher than of the productivities of other fungi such as *Aspergillus brasiliensis* CFF124 (0.0729 U/mLh) (Zaghetto de Almeida et al., 2024a), *Aspergillus oryzae* F-923 (0.118 U/mLh) (Fadel et al., 2020), *Aspergillus oryzae* LZ2 (0.120 U/mLh) (C. Wang et al., 2020), among others, as shown in Table 6. It was only surpassed by the enzyme produced by *Aspergillus niger* van Tieghem IBRC-M 30064 (0.268

U/mL*h)(Reihani et al., 2024), demonstrating that it is possible to obtain an enzyme with high activity in a short fermentation period. It is suggested that milled corn may also contain inducers of enzymatic metabolism, components that are not present in starch, wheat bran starch, maltose, and fructose, as the highest enzymatic activities were obtained with this substrate (Table 6).

To evaluate the impact of forced aeration on enzymatic production, MDL-750-10L fermenter, brand B.E. Marubishi (1327) was used. Unlike Erlenmeyer flasks, which rely on diffusion aeration, the STR features a forced aeration system that optimized productivity time, as controlled agitation and aeration significantly enhance oxygen and mass transfer while ensuring more efficient medium homogenization, key conditions to accelerate microbial metabolism. The highest enzymatic activity in the STR was observed on the third day of fermentation, whereas in the Erlenmeyer flasks it occurred on the fifth day. Figure 7, in the first fermentation without antifoam oil, the maximum activity was 21.64 U/mL. By initially adding 5 mL of antifoam oil, a maximum activity of 18.37 U/mL was achieved. In a third trial, with optimization of the antifoam oil addition, a maximum activity of 24.96 U/mL was reached.

The data analysis showed a decrease in β-glucoamylase activity in the bioreactor compared to the Erlenmeyer flasks, from 27.20 U/mL to 24.96 U/mL, attributed to differences in mass transfer or shear force generated by the agitators on the *Aspergillus oryzae* mycelium. Since *Aspergillus oryzae* is a filamentous fungus, it can suffer growth damage from direct agitation. To mitigate this issue, a bubble column bioreactor (BCR) could be considered as an alternative, as it provides high oxygen transfer efficiency without mechanical agitation, thereby reducing shear stress, which is crucial for fungal growth (Valladares-Diestra, K.K., 2021). Several studies have demonstrated the advantages of BCRs in enzyme production using filamentous fungi, as they enhance biomass yield while maintaining fungal morphology (Valladares-Diestra, K. K. et al., 2022). Further research could evaluate the feasibility of using a BCR for β-glucoamylase production.

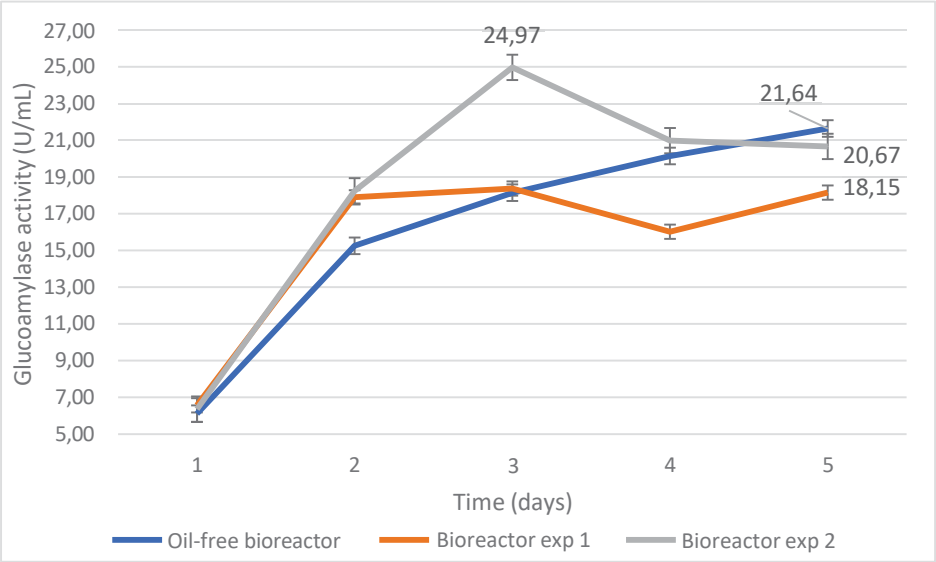

Fig. 7: Time course profile of β-glucoamylase production by *Aspergillus oryzae* NRRL1808 in STR

4.3.4. *Recovery, Separation, and Purification of β-glucoamylase Using Microfiltration, Ultrafiltration, and SDS-PAGE Analysis*

The enzymatic extract was processed by microfiltration and ultrafiltration, showing enzymatic activity, unlike the filtrate from the 30 kDa membrane, where no activity or proteins were detected. Fungal β-glucoamylases typically have molecular sizes between 60-86 kDa (K. Gupta & Nagar, 2020; Lago et al., 2021) which supports the presence of β-glucoamylase in this study. The highest enzymatic activity (41 U/mL) was observed in the 30 kDa retentate, with a specific activity of 2800 U/mg (Table 7).

SDS-PAGE results indicated that the molecular weight of the obtained β-glucoamylase was between 60-65 kDa. This molecular weight range is consistent with values reported in the literature (Table6): 60 kDa (K. Gupta & Nagar, 2020; C. Wang et al., 2020), 65 kDa (Fadel et al., 2020), 69 kDa (Zaghetto de Almeida et al., 2024a), 70 kDa (Reihani et al., 2024), and 86 kDa (Lago et al., 2021). Additionally, gel images confirmed that β-glucoamylase was the only protein produced by the fungus, suggesting that the optimized medium exclusively induced the production of β-glucoamylase by *Aspergillus oryzae* LPB1808 (Gomes et al., 2005).

Table 7. Purification and concentration of β-glucoamylase using micro and ultrafiltration membranes in the Vivaflow 200 system.

Purification steps	β-glucoamylase (U/mL)	Protein (mg/mL)	Volume (mL)	Total Activity (U)	Total Proteins (mg)	Specific Activity (U/mg)	Purification fold	Yield
Optimum point	55,94	0,035	650	36361,845	22,75	1598,322857	1	100%
Microfiltration 1: Filtrate	34,55	0,022	245,00	8463,73	5,34	1584,63	0,99	99.14%
Microfiltration 1: Retained	28,77	0,020	140,00	4028,23	2,83	1421,82	0,89	88.95%
Ultrafiltration (100KDa): Filtrate	24,01	0,014	120,00	2881,26	1,71	1688,20	1,06	105.62%
Ultrafiltration (100KDa): Retained	30,87	0,028	100,00	3086,57	2,76	1116,93	0,70	69.88%
Ultrafiltration (30KDa) : Filtrate	0	0	20,00	0	0	0	0	0%
Ultrafiltration (30KDa) : Retained	41,00	0,015	80,00	3280,33	1,17	2800,16	1,75	175.19%

4.3.5. *Characterization of β-glucoamylase and definition of optimal parameters for enzymatic activity*

Thanks to the one factor at a time (OFAT) optimization, the results were analyzed, demonstrating that the optimal pH for β-glucoamylase activity is 5, achieving an activity of 47.51 U/mL (Figure 8(A)). Results at different temperatures showed that the maximum enzymatic activity was observed at 55°C, with a value of 46.72 U/mL (Figure 8(B)).

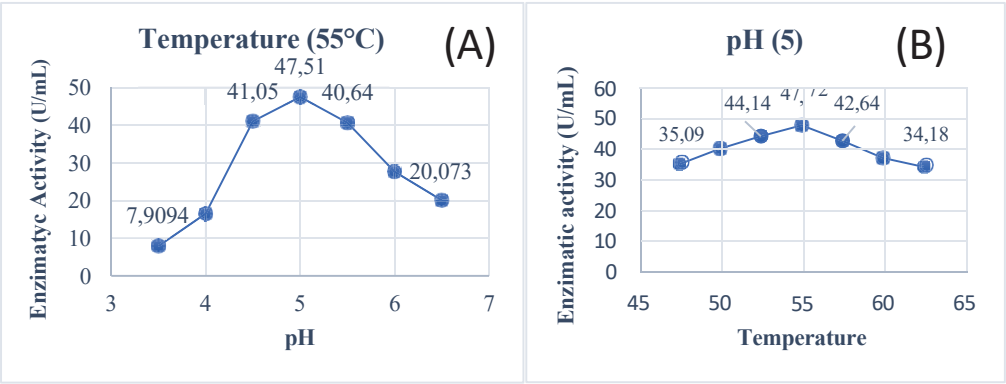

Fig. 8: β-glucoamylase activity of *A. oryzae* LPB1808 (A) at different pH levels and (B) at different temperatures.

A central composite rotational design (CCRD) was used to study the interaction between temperature (55°C) and pH (5), with the goal of optimizing the enzymatic activity parameters (Fig. 9). The F-value of the quadratic model was 6.03, and the coefficient of determination (R^2) was 0.8578. The resulting Equation 2 predicted that the theoretical maximum enzymatic activity would be 47.8822 U/mL at a temperature of 53.87°C and a pH of 5.335. Under these conditions, the experimentally obtained enzymatic activity was 47.625 U/mL, representing a 100.54% improvement over the predicted values.

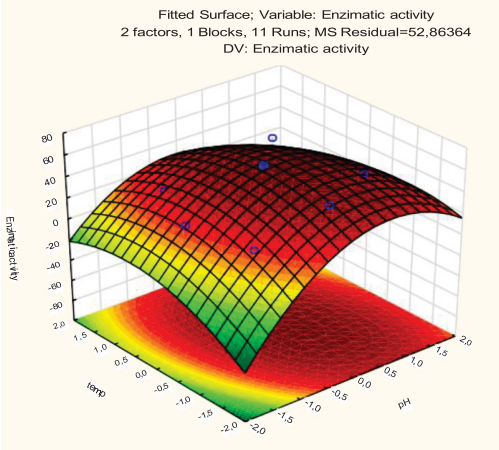

Fig.9: Response surface of CCDR to determine optimal pH and temperature values for β-glucoamylase.

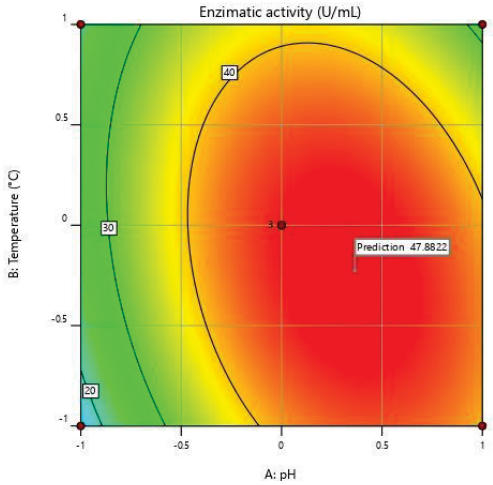

Fig.10: Response surface of the optimized prediction for β-glucoamylase activity.

$$Glucoamylase\ activity\ (\frac{U}{mL}) = 56.45 + 7.65 * A - 1.51 * B - 4.68 * AB - 13.18A * (A^2) - 6.42 * (B^2). (Equation\ 2)$$

According to (Zaghetto de Almeida et al., 2024a), β-glucoamylase have an operational temperature range between 45 and 65°C, and a pH range between 4 and 6. After analyzing the data from the proposed mathematical model, it was determined that the optimal activity of the β-glucoamylase produced by *Aspergillus oryzae* LPB1808 occurs at 53.85°C and a pH of 5.3 (Figure 10). This temperature range is lower than the optimal point of 60°C and pH 5 reported by (Reihani et al., 2024; Zaghetto de Almeida et al., 2024), highlighting the low thermotolerance of the enzyme produced in this study. This suggests that the enzyme loses its functional activity at extreme temperatures, leading to protein denaturation or inactivation.

4.3.6. *Formulation of the β-glucoamylase enzymatic extract.*

After 15 days of storage, the residual activities obtained were 93.03% for the formulated extract stored at 4°C and 81.14% for the formulation kept at room temperature (20°C) (Figure 11). The residual activity of the formulated extract stored at 55°C was discarded due to the rapid decline in activity from day 5 (18.25%) to day 15 (7.44%). These results suggest that, at 55°C, the enzyme completely loses its activity, indicating irreversible thermal denaturation. In contrast, the residual activities of the unformulated enzymatic extracts stored under refrigeration and at room temperature were 79.74% and 70.77%, respectively.

These results highlight the relevance and effectiveness of using additives for preserving enzymatic activity. The data obtained in this study were compared to those reported by Silva, (2017), who observed a residual activity above 90% at 15 days for amylolytic enzymes obtained and formulated; the incorporation of additives in the β-glucoamylase formulation proved to be highly beneficial, as enzymes, in general, may become more susceptible to degradation after purification processes than in their natural form. Maintaining the activity of an enzymatic formulation for 15 days at levels close to 93.03% represents an excellent result and an interesting approach for evaluating its stability over longer periods.

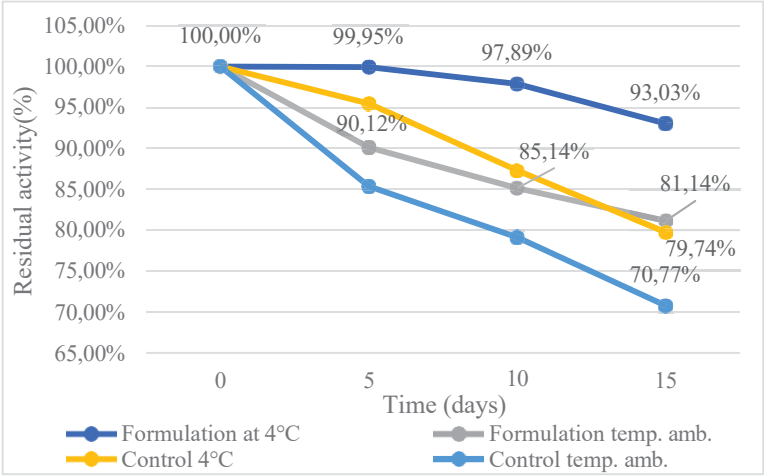

Fig.11: Residual β-glucoamylase activity comparing samples formulated with additives under refrigeration and without formulation

4.3.7. *Application of β-glucoamylase for the release of total reducing sugars from agro-industrial product (Milled corn).*

The metabolism of a fungal β-glucoamylase produced in the laboratory was evaluated and compared with that of a commercial β-glucoamylase (Figure 12) to determine the efficiency of both enzymes in the conversion of maltose to glucose. The resulting hydrolysate was analyzed using a High-Performance Liquid Chromatography (HPLC) system, equipped with an Aminex HPX-87H column, maintained at 65°C, with a mobile phase composed of a 5 mM sulfuric acid solution and a flow rate of 0.6 mL/min, to identify the sugars present (Alt et al., 2024; Zaghetto de Almeida et al., 2024). The results obtained allowed the identification of the metabolic profile of the produced β-glucoamylase, confirming its classification as β-glucoamylase due to the similarity in metabolic patterns observed during hydrolysis. The commercial enzyme achieved a higher final glucose yield (66.90 g/L), compared to the experimental enzyme (28.97 g/L). This finding suggests that the differences in total glucose yield may be attributed to factors such as concentration, stability, optimized specific activity, or the presence of activating ions in the commercial enzyme. The laboratory-produced β-glucoamylase followed the same metabolic mechanism, generating 0.232 g of glucose per gram of hydrolyzed milled corn, while the commercial enzyme produced 0.534 g of glucose per gram of milled corn. These results indicate that the fungal enzyme exhibits a yield of 43.44% compared to the commercial version, which is promising for future optimizations aimed at improving enzyme performance. These results highlight the need to continue refining the production and purification conditions of fungal β-

glucoamylase in order to match or even surpass the efficiency of commercially available versions.

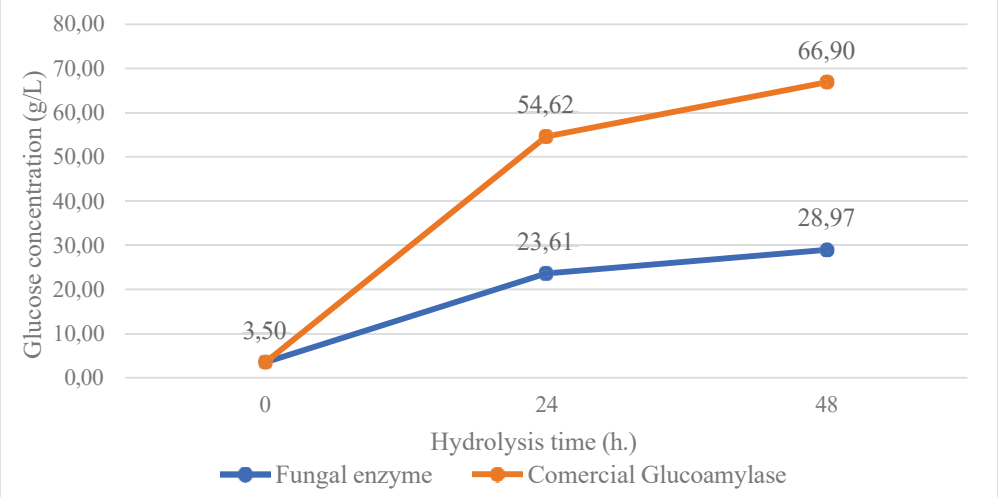

Fig. 12: Enzymatic metabolism in the hydrolysis of maltose: A comparison between commercial β-glucoamylase and produced fungal β-glucoamylase.

4.4. Conclusion

This study developed an innovative and efficient process to optimize the production of β-glucoamylase from the fungus *Aspergillus oryzae* LPB1808, utilizing milled corn solids in suspension within a submerged fermentation (SmF) system. This agro-industrial product, abundant in agricultural countries like Brazil, proved to be a cost-effective alternative that not only reduced production costs but also provided a suitable nutrient source for fungal growth and enzymatic production. Through qualitative and quantitative evaluations, *Aspergillus oryzae* LPB1808 was confirmed as an efficient β-glucoamylase producer, and during the optimization phase, enzymatic activity increased from 14.29 U/mL to 41.00 U/mL, representing a 186.89% improvement compared to the initial level. Following the characterization of optimal conditions, enzymatic activity further reached a maximum value of 47.625 U/mL. Additionally, kinetic analysis conducted in Erlenmeyer flasks and a stirred-tank reactor (STR) identified the best conditions to maximize production while highlighting critical considerations for process scaling. The recovery and partial purification of the enzyme using microfiltration and ultrafiltration membranes ensured a high-quality product suitable for industrial applications. Moreover, SDS-PAGE analysis estimated the enzyme's molecular weight at 60–65 kDa, while ions such as Ca^{+2}, Mn^{+2}, Cu^{+}, and Mg^{+2} were found to enhance its production. However, the enzyme demonstrated sensitivity to variations in pH and temperature, emphasizing the necessity for additional stabilization strategies. To address this, additives like Solutions A and B were proposed,

preserving and stabilizing amylolytic activity at 93.03% after 15 days of storage. The β-glucoamylase showed its efficacy in converting corn starch into glucose (28.97 g/gds), underscoring its potential application in biofuel production, particularly bioethanol, achieving a yield of 43.44% compared to a commercial β-glucoamylase. These findings not only present a promising outlook for the sustainable production of β-glucoamylase with *Aspergillus oryzae* LPB1808 but also establish a foundation for formulating liquid enzymatic complexes and evaluating their stability under various storage conditions, thus facilitating their future application in industrial processes while contributing to the reduction of the environmental impact derived from agro-industrial products.

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5. CONSIDERAÇÕES FINAIS

A otimização da produção de glucoamilases com *Aspergillus oryzae* LPB1808, usando milho moído como fonte de carbono, obteve resultados satisfatórios por meio de análises estatísticas. No entanto, o metabolismo fúngico pode ser influenciado por fatores como características do substrato e disponibilidade de amido. Por isso, a produção de glucoamilases com fungos exige análises físico-químicas adicionais para alcançar a padronização. As glucoamilases, embora amilolíticas, possuem composição variada, com diferentes tamanhos e pesos moleculares. Fungos do gênero *Aspergillus* são grandes produtores de enzimas industriais devido à sua adaptabilidade em ambientes ricos em compostos orgânicos.

6. RECOMENDAÇÕES PARA TRABALHOS FUTUROS

Como considerações finais, é importante identificar até que ponto esta pesquisa conseguiu desenvolver uma enzima capaz de ser utilizada para a produção industrial de bioetanol, utilizando o milho moído, como fonte de produção. Nesse sentido, destacamos que para futuras investigações com o mesmo enfoque, é necessário:

- Determinar quimicamente a composição da enzima produzida. Utilizando tecnologias como MALDI-TOF/TOF, cristalografia de raios X ou espectroscopia de ressonância magnética nuclear (RMN), seria possível fragmentar a enzima e identificar os aminoácidos que a compõem, o que poderia influenciar sua atividade, para projetos futuros, compreender como potencializar essa atividade é essencial.
- Realizar experimentos com a sacarificação e fermentação simultânea. Esse tipo de tecnologia não foi explorado devido à limitação de tempo, mas é amplamente relatado, especialmente em aplicações industriais de glucoamilases destinadas à produção de bioetanol.
- Desenvolver estudos sobre a interação do fungo com diferentes produtos agroindustriais, com o objetivo de avaliar os efeitos positivos ou negativos que estes possam ter sobre sua produção enzimática.
- Experimentar com outros compostos para a formulação dos complexos enzimáticos, que possam conservar melhor a atividade da glucoamilase estudada e que não interfiram em sua atividade final.

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