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Review

Potentials of Filamentous Fungi for the Second Generation of Bioethanol Production: Recent Advances, Enzymatic Strategies, and Biorefinery Integration

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Abstract

The increasing global demand for sustainable energy has intensified interest in bioethanol as a renewable alternative to fossil fuels. While first- and second-generation bioethanol technologies have advanced considerably, challenges related to production cost, efficiency, and sustainability remain. Filamentous fungi have emerged as promising biocatalysts due to their ability to produce cellulolytic and hemicellulolytic enzymes that efficiently degrade lignocellulosic biomass into fermentable sugars. Genera including *Trichoderma*, *Aspergillus*, *Fusarium*, *Neurospora*, and *Penicillium* play significant roles in enhancing biomass saccharification and improving ethanol production. Co-cultivation strategies involving filamentous fungi and yeasts further enhance substrate utilization, ethanol yield, and process stability, although scalability and culture compatibility remain challenging. Recent advances in metabolic engineering, mutagenesis, and CRISPR-based genome editing have enabled the development of improved fungal strains with enhanced enzymatic performance and substrate conversion efficiency. Beyond bioethanol production, filamentous fungi are increasingly recognized as key components of integrated biorefinery systems through their capacity to utilize diverse feedstocks and generate value-added products, including organic acids, industrial enzymes, and bioactive compounds. Despite limitations such as variable growth rates, substrate heterogeneity, and inhibitory by-products, filamentous fungi represent versatile and sustainable platforms for advancing second-generation bioethanol production and supporting future circular bioeconomy and biorefinery development.

Keywords: biorefinery; filamentous fungi; lignocellulosic biomass; metabolic engineering; second-generation bioethanol

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1. Introduction

The rapid pace of industrialization, urbanization and population growth has intensified the global energy crisis and highlighted the overreliance on non-renewable energy resources [1]. Since energy is essential for both societal progress and daily continuity, projections indicate a sharp increase in demand by 2030 with fossil fuel reserves potentially being depleted by 2060 [2]. Rising oil prices and escalating global energy consumption have therefore compelled many nations to explore alternative energy options, particularly biofuels [3,4]. Biofuels are increasingly recognized as a low-carbon substitute for fossil fuels because they can reduce greenhouse gas emissions and mitigate climate change impacts

associated with transport [5]. When blended with conventional fuels, biofuels also enhance fuel properties, lowering tailpipe emissions and improving air quality [6]. In addition, supportive policies, emission reduction targets and advances in crop engineering and fermentation technologies have accelerated their development [7]. Among the various biofuels under development, including biogas, biodiesel, biohydrogen and biobutanol, ethanol remains the most widely used [8].

The global bioethanol market has expanded steadily, recording a compound annual growth rate of 7.6% between 2016 and 2022, with distribution varying according to feedstock, blending type, such as E5, E10 and E85, vehicle application and regional market demand [9]. Ethanol is typically blended with gasoline, with mixtures such as E85 containing 85% ethanol and 15 percent gasoline proven to reduce greenhouse gas emissions [10]. Production reached a peak of 115 billion liters in 2019, but the COVID-19 pandemic caused a significant decline in 2020 due to reduced transport fuel consumption and profitability challenges [11]. Global demand for biofuels is forecasted to grow by approximately 38 billion liters between 2023 and 2028, marking an increase of nearly 30% over the previous five-year period. By 2028, total consumption is expected to approach 200 billion liters, with ethanol and renewable diesel accounting for about two-thirds of this expansion [12]. While ethanol as a fuel is not a novel concept since it was already used at the beginning of the twentieth century to power Henry Ford's Model T, it is now viewed as a key substitute for petroleum-derived fuels because it is derived from renewable raw materials, is less toxic than other alcohol-based fuels such as butanol, can be blended directly with gasoline in varying ratios, reduces pollutant emissions due to its inherent oxygen content and produces less toxic by-products upon combustion [13].

Industrial bioethanol production is generally categorized into three generations based on the feedstock used. First-generation bioethanol (1G) relies on food crops such as sugarcane and corn, which have long dominated the industry but face criticism for competing with food resources [14]. Second-generation bioethanol (2G), derived from non-edible lignocellulosic residues, offers the dual benefits of managing agricultural waste and lowering greenhouse gas emissions [15]. However, the recalcitrant structure of lignocellulose formed by tightly bound cellulose, hemicellulose and lignin poses challenges to efficient degradation [16]. Third-generation bioethanol (3G) focuses on microalgae as feedstock, which is considered promising due to its ability to fix carbon dioxide from air and flue gases, achieve higher solar energy conversion efficiency than terrestrial plants and generate biomass rich in proteins, lipids and carbohydrates [17,18]. Although third-generation technology is still at an early stage and contributes less to current bioethanol production, ongoing research seeks to identify and engineer algal strains with higher productivity [19].

At present, *Saccharomyces cerevisiae* remains the dominant microorganism in industrial ethanol production owing to its robust fermentation capacity, tolerance to stress and ability to achieve yields close to the theoretical maximum [20]. Nevertheless, its inability to efficiently metabolize pentose sugars such as xylose has driven genetic engineering efforts to expand its substrate utilization [21]. Filamentous fungi, including *Neurospora crassa*, *Mucor* spp., *Fusarium oxysporum*, *Monilia* spp. and *Paecilomyces* spp., provide an alternative since they can ferment pentoses and directly utilize plant-derived substrates, which is generally absent in yeast systems [22].

Filamentous fungi are also prolific producers of extracellular enzymes that depolymerize complex biomass into simple sugars, which can then be converted into bioethanol or other value-added products by the same organism or in co-culture systems [23]. Industrially relevant genera include *Aspergillus*, *Rhizopus*, *Trichoderma*, *Penicillium*, *Fusarium* and *Neurospora* [24]. Many of these fungi can metabolize a wide spectrum of hexose and

pentose sugars. For instance, *Trichoderma reesei* is widely used at the commercial scale for cellulase and hemicellulase production [25]. While cellulolytic fungi are effective at producing enzymes, their ethanol fermentation efficiency is lower than that of *S. cerevisiae* [23]. This limitation arises partly because they are primarily aerobic organisms, which hinders their performance under anaerobic fermentation conditions [16]. These constraints underscore that the industrial competitiveness of filamentous fungi in second-generation bioethanol production depends on overcoming several interconnected bottlenecks, including efficient lignocellulose deconstruction, tolerance to pretreatment-derived inhibitors, balanced enzyme production, high ethanol productivity, genetic tractability, and process scalability. Rather than considering these factors independently, they should be viewed as integrated determinants of successful industrial implementation. Accordingly, this review critically evaluates recent advances in fungal pretreatment, physiology, enzymatic systems, co-culture strategies, metabolic engineering, CRISPR-based genome editing, and biorefinery integration in the context of how each addresses these bottlenecks. The review also identifies the remaining scientific and technological challenges that must be overcome to enable the commercial deployment of filamentous fungi for sustainable second-generation bioethanol production. The conceptual framework guiding this review is illustrated in Figure 1.

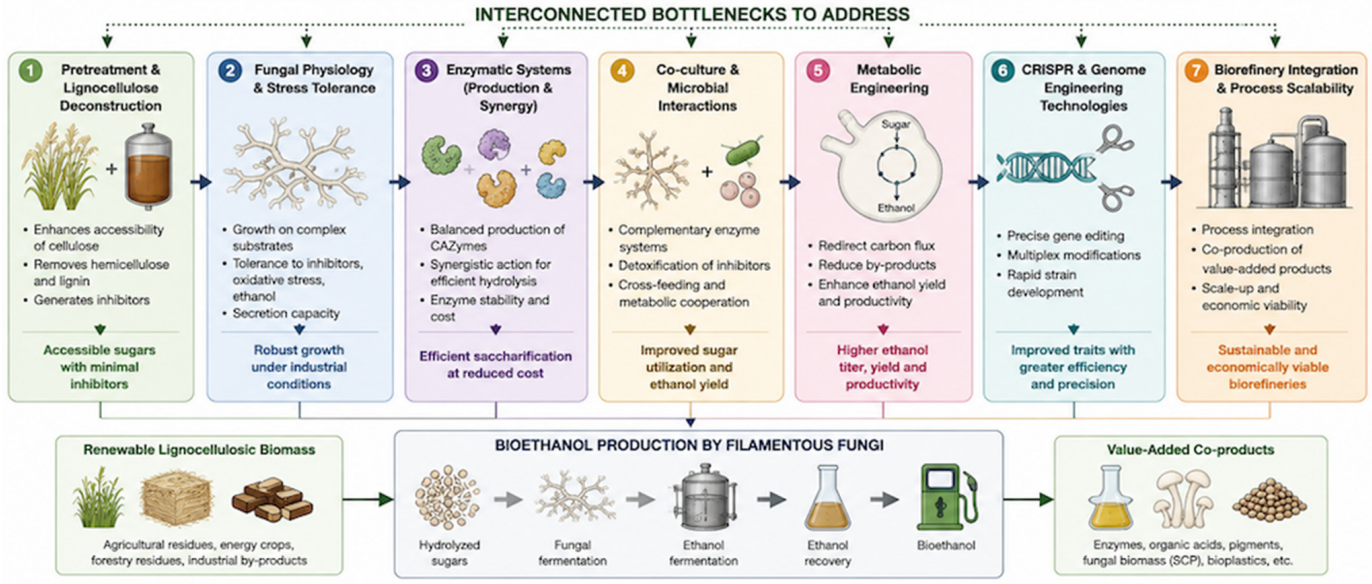


Figure 1. Conceptual framework illustrating six interconnected bottlenecks that determine the industrial competitiveness of filamentous fungi in second-generation bioethanol production.

2. Methodology

This review was conducted through a comprehensive literature search to gather recent information and analytical data on the application of filamentous fungi in bioethanol production and their emerging roles in biorefinery systems. The search strategy included multiple electronic databases such as Google Scholar, Scopus, ScienceDirect, and PubMed to ensure wide coverage of relevant and high-quality publications. Articles published between 2020 and 2025 were prioritized to reflect the most recent advances, validated findings, and updated perspectives in the field.

The main keywords applied were “bioethanol production”, “lignocellulosic biomass”, “filamentous fungi”, “co-culturing”, “metabolic engineering”, and “biorefinery applications”. Boolean operators “AND” and “OR” were used to refine and broaden the search, while backward and forward citation tracking was employed to identify additional studies not captured in the initial search.

Selection of publications was guided by explicit inclusion and exclusion criteria. Studies were included if they addressed (i) the role of filamentous fungi in lignocellulosic biomass degradation and ethanol production, (ii) co-culture strategies involving fungi and yeasts, (iii) recent genetic or metabolic engineering approaches for enhancing fungal performance, and/or (iv) prospects of filamentous fungi within integrated biorefinery frameworks. Publications outside these areas, duplicates, non-peer-reviewed sources, and non-English articles were excluded.

Comparative assessment of selected studies was carried out to evaluate strengths, limitations, and practical applications, with a particular focus on enzyme efficiency, fermentation strategies, and industrial scalability. This review adopts a theoretical analysis framework to synthesize findings, critically assess current progress, and highlight research gaps, consistent with the approach described by [26].

3. Lignocellulosic Biomass and Mechanisms of Filamentous Fungi in Bioethanol Production

Lignocellulosic biomass represents the main feedstock for second-generation (2G) bioethanol production, providing a sustainable alternative to food-based materials used in first-generation technologies. Growing concerns about food versus fuel competition have redirected attention toward non-edible plant sources and agricultural residues that are abundant and cost-effective [27]. Typical feedstocks include non-food crops such as *Jatropha curcas* and switchgrass, as well as agro-industrial by-products like stems, straw, husks, and leaves [28].

From a compositional perspective, lignocellulosic biomass consists of approximately 40–60% cellulose, 20–40% hemicellulose, and 10–25% lignin, with smaller portions of proteins, ash, and extractives [29]. The intricate bonding between these polymers through covalent, hydrogen, and van der Waals interactions contributes to the material's high recalcitrance, thus requiring pretreatment to facilitate enzymatic hydrolysis [19].

Among biological pretreatment agents, filamentous fungi play a vital role due to their ability to secrete enzymes that deconstruct plant cell wall polymers. These fungi produce cellulases, hemicellulases, and lignin-modifying enzymes that act synergistically to convert cellulose and hemicellulose into fermentable sugars. Cellulose, which generally accounts for 30–50% of the dry weight of lignocellulosic biomass, serves as the primary substrate for glucose production [30]. Its linear β-1,4-linked glucose chains form crystalline microfibrils interspersed with amorphous regions, where enzymatic activity is typically initiated [31,32]. Regions of high crystallinity resist enzymatic attack, whereas amorphous areas are more accessible, allowing cellulolytic fungi to initiate hydrolysis effectively [33,34].

Hemicellulose, a heteropolymer composed of pentose and hexose sugars such as xylose, mannose, and galactose, complements cellulose degradation by providing additional fermentable sugars [35]. Due to its branched and acetylated structure, hemicellulose is less crystalline and thus more susceptible to fungal hydrolysis [36,37]. In hardwoods, xylan constitutes about 35% of hemicellulose, while glucomannan dominates in softwoods, contributing approximately 28% [38,39].

Lignin, a complex phenolic polymer comprising guaiacyl, syringyl, and p-hydroxyphenyl units, functions as a structural binder between cellulose and hemicellulose [40]. Its high chemical stability and hydrophobic nature impede enzyme access, making delignification a critical step in biomass processing [41]. A higher lignin content is typically associated with reduced cellulose accessibility and lower sugar yield [42,43]. Current approaches focus on modifying lignin composition and developing efficient pretreatment methods that complement fungal enzymatic degradation [44].

The structural complexity of lignocellulosic biomass, which results from the combined presence of cellulose, hemicellulose, and lignin, poses a significant challenge for second-generation biofuel production. To overcome this barrier, filamentous fungi deploy a comprehensive enzymatic system. The successful conversion of lignocellulose into fermentable sugars requires the synergistic activity of cellulases that act on both soluble and insoluble cellulose chains while contributing to hemicellulose breakdown [45,46]. These enzymes, classified as glycoside hydrolases, specifically cleave the β-1,4 glycosidic bonds within cellulose and related oligosaccharides, transforming them into glucose units [47]. During enzymatic hydrolysis, the structural carbohydrates, including cellulose, hemicellulose, and starch, are degraded, releasing pentose and hexose sugars that serve as substrates for ethanol fermentation [48].

From a technological perspective, the conversion efficiency of lignocellulosic biomass largely depends on its compositional ratio and the enzymatic capacity of the fungal strain employed. Substrates with a high cellulose fraction, such as sugarcane bagasse and rice straw, typically produce higher ethanol yields than lignin-rich residues due to better enzyme accessibility and reduced structural recalcitrance. Establishing correlations between biomass structure and fungal enzyme activity enables the identification of feedstocks that maximize saccharification efficiency and enhance the overall return on investment (ROI) in second-generation bioethanol production.

The economic viability of lignocellulosic bioethanol manufacturing is determined by several interrelated factors, including feedstock price, enzyme cost, co-product generation, and process integration [49]. Among these, enzyme production and utilization represent a major cost component, making the optimization of enzyme efficiency and recovery crucial for achieving commercial competitiveness. In this context, several techno-economic analyses have suggested that an effective strategy for minimizing production costs is the implementation of on-site cellulase synthesis, which eliminates the need to purchase costly commercial enzyme preparations. Consequently, this approach has been shown to reduce overall enzyme-related expenses by approximately 30–70%, depending on the process scale and fermentation efficiency [50].

Furthermore, optimization of fermentation parameters is essential for enhancing cellulolytic and ligninolytic enzyme productivity, as it shortens fermentation duration, reduces energy consumption for temperature regulation, and decreases the demand for expensive culture-medium components [51]. A comprehensive understanding of these biochemical and process-level interactions forms the foundation for developing effective enzyme cocktails and optimizing fungal systems for industrial-scale bioethanol manufacturing.

3.1. Hydrolytic Enzymes for Biodegradation of Lignocellulosic Biomass

The hydrolysis of cellulose represents a crucial stage in biomass utilization, requiring the coordinated action of multiple enzymes to overcome the challenges associated with degrading such a highly recalcitrant substrate [52]. Cellulases are modular enzymes composed of distinct domains that allow them to function in a complementary manner, facilitating the breakdown of insoluble cellulose into soluble oligosaccharides [53]. These enzymes belong to the glycosyl hydrolase family and consist primarily of three major components, namely exoglucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4), and β-glucosidase (EC 3.2.1.21) [54]. Together, they act in synergy to hydrolyze cellulose completely into glucose, as illustrated in Figure 2.

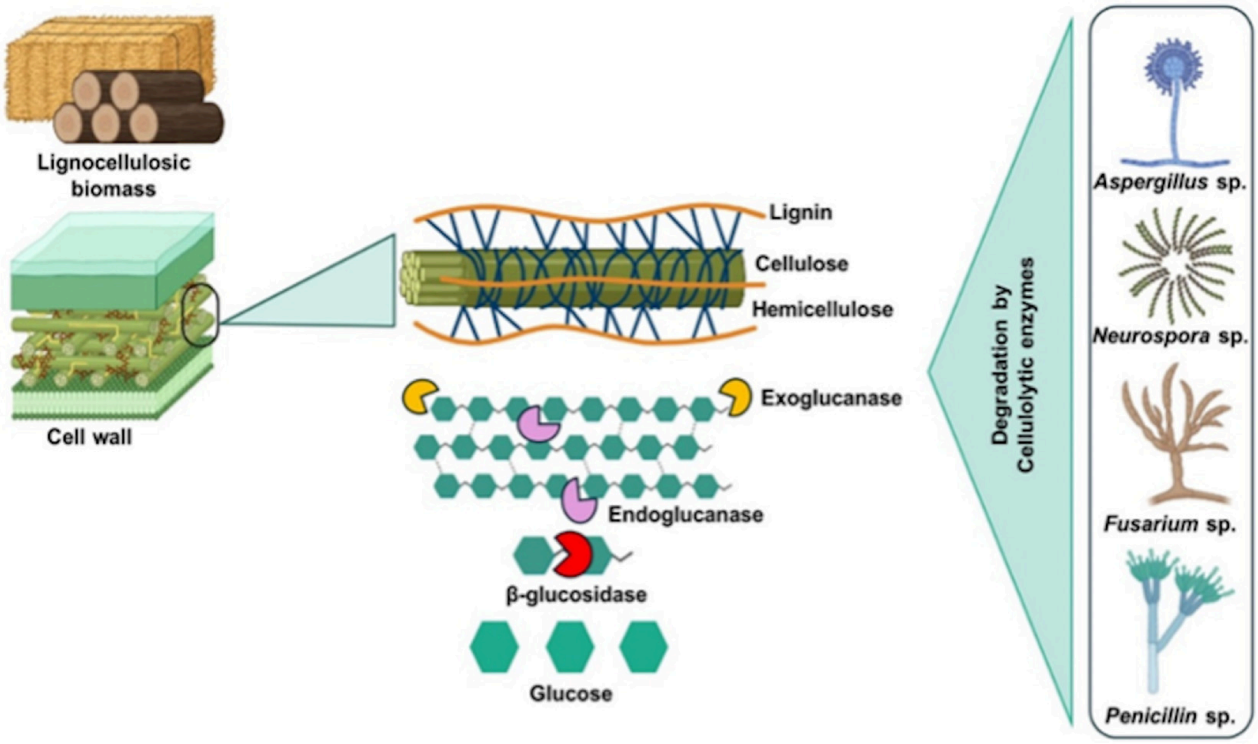


Figure 2. Cellulose degradation from lignocellulosic biomass into glucose by cellulolytic enzymes produced by filamentous fungi.

3.1.1. Endoglucanase (EC 3.2.1.4)

Endoglucanases (EGs) play a critical initiating role in cellulose depolymerization by randomly hydrolyzing internal β-1,4-glycosidic bonds within amorphous regions of cellulose microfibrils, producing shorter cellodextrins, cellobiose, and glucose. Unlike exoglucanases, which predominantly act on crystalline cellulose by removing cellobiose units from chain ends, endoglucanases enhance cellulose accessibility by disrupting internal fiber structures and generating new chain termini for subsequent enzymatic attack [55]. This synergistic interaction between EGs and other cellulolytic enzymes is essential for efficient lignocellulosic biomass degradation. However, their industrial application remains challenged by the limited accessibility of highly crystalline cellulose, inhibition by lignin-derived compounds, reduced stability under harsh processing conditions, and the high cost associated with enzyme supplementation. Advances in enzyme immobilization and formulation technologies have improved enzyme stability, recovery, and reusability, thereby increasing the feasibility of EG application in lignocellulosic bioconversion processes [56].

In addition to process optimization, molecular engineering strategies have emerged as promising approaches to enhance EG performance. Directed evolution, rational mutagenesis, and structure-guided engineering have been employed to improve catalytic efficiency, thermostability, pH tolerance, and resistance toward inhibitors generated during biomass pretreatment. Structural studies of glycoside hydrolases have revealed that modifications within catalytic domains, substrate-binding regions, surface loops, and flexible linker sequences can significantly influence substrate interaction and hydrolytic efficiency. Engineering of carbohydrate-binding modules (CBMs) has also received considerable attention, as improved cellulose recognition and enzyme positioning on insoluble substrates can enhance catalytic effectiveness while minimizing unproductive binding to lignin-rich fractions [57]. Furthermore, the incorporation of auxiliary oxidative enzymes such as lytic polysaccharide monooxygenases (LPMOs) has expanded current concepts of cellulose

degradation. For example, fungal AA9 LPMOs with different regioselectivities (C1, C4, or C1/C4 oxidation) and domain structures (with or without a CBM1) have been shown to oxidize natural Cellulose I fibers and, in some cases, Cellulose II, with CBM-lacking variants producing more dispersed oxidation patterns and higher overall fiber oxidation, thereby enhancing accessibility for classical hydrolytic enzymes [58].

Filamentous fungi represent particularly valuable platforms for EG production due to their natural capacity for extracellular enzyme secretion, broad substrate utilization, and coordinated expression of cellulolytic gene networks. In industrially relevant fungi such as *T. reesei*, *Aspergillus* spp., and *Penicillium* spp., genetic engineering approaches targeting transcriptional regulators, promoter systems, and secretion pathways have been used to improve cellulase production and enzyme cocktail composition. For example, overexpression of the major cellulase regulator *xyr1* in *T. reesei* has been demonstrated to enhance the expression of cellulolytic enzymes, supporting more efficient conversion of lignocellulosic residues into fermentable sugars [59]. When integrated into microbial consortia, engineered fungal strains can complement other microorganisms by supplying hydrolytic enzymes in situ, reducing dependence on externally produced enzyme preparations and enabling more efficient consolidated bioprocessing strategies for bioethanol production.

Recent advances have further shifted cellulase engineering from conventional screening approaches toward predictive and rational design. Computational tools, including molecular docking, molecular dynamics simulations, and machine learning-based prediction platforms, enable the identification of structural determinants associated with improved enzyme activity, substrate specificity, and environmental tolerance. Similarly, recombination strategies such as SCHEMA-based engineering have facilitated the generation of chimeric cellulases with enhanced catalytic properties compared with their parental enzymes [53]. Collectively, these developments highlight that fungal endoglucanases should no longer be considered static hydrolytic enzymes but rather as adaptable biological components that can be optimized through enzyme engineering, fungal strain improvement, and synergistic enzyme system design. Such integrated approaches will be crucial for maximizing fermentable sugar recovery and improving the economic feasibility of second-generation bioethanol production.

3.1.2. Exoglucanase (EC 3.2.1.91)

Exoglucanases, also known as cellobiohydrolases (CBHs), are secreted by microorganisms to hydrolyse cellulose chains from their reducing or non-reducing ends. These enzymes cleave β-1,4-glycosidic bonds at the termini of cellulose microfibrils, releasing primarily cellobiose together with small amounts of glucose [60]. A defining characteristic of CBHs is their high processivity, which enables continuous movement along cellulose chains while maintaining substrate contact, thereby efficiently dismantling crystalline cellulose fibrils into soluble products [61]. Owing to their unique ability to degrade highly ordered cellulose, CBHs constitute a major component of commercial cellulase cocktails for lignocellulosic biomass conversion [62]. In *Trichoderma reesei*, CBH1 (Cel7A) and CBH2 (Cel6A) collectively account for more than 70% of the secreted cellulase proteins and are responsible for the majority of crystalline cellulose hydrolysis [53]. In contrast, brown rot fungi generally lack CBHs and instead rely on oxidative, non-enzymatic mechanisms coupled with endoglucanase activity to depolymerize cellulose [63].

Recent studies have expanded exoglucanase engineering beyond individual protein optimization toward systems-level strategies for improving cellulase production. Multiomics analyses of cellulolytic fungi, including *Trichoderma guizhouense*, have enabled the identification of coordinately expressed exoglucanases and associated accessory proteins

involved in lignocellulose degradation. These datasets provide valuable resources for identifying candidate exoglucanases with desirable structural features, including catalytic tunnel architecture, substrate-binding loops, carbohydrate-binding modules (CBMs), flexible linker regions, and glycosylation patterns that collectively determine processivity, substrate affinity, and enzyme stability. Rational mutagenesis, directed evolution, and glycoengineering have subsequently been employed to improve catalytic efficiency, thermostability, secretion efficiency, and tolerance toward inhibitory compounds and non-productive adsorption associated with lignocellulosic substrates under industrial saccharification conditions [64].

Complementing structural studies, systems biology approaches such as gene regulatory network (GRN) reconstruction have demonstrated that exoglucanase production is governed by coordinated transcriptional programs rather than isolated gene regulation. GRN analyses in *Penicillium echinulatum* and *Penicillium oxalicum* have identified key transcription factors and regulatory modules controlling cellobiohydrolase expression, demonstrating that CBH production is regulated through complex genetic networks. These findings provide potential targets for transcription-factor engineering, promoter rewiring, and rational strain development to enhance cellulase production [65]. Together with computational approaches, including molecular dynamics simulations, structure-guided protein engineering, and machine learning-assisted prediction of beneficial mutations, these advances are accelerating the development of next-generation exoglucanases with improved catalytic performance, enabling more efficient enzyme cocktails for lignocellulosic biomass conversion and bioethanol production.

3.1.3. β-Glucosidase (EC 3.2.1.21)

β-glucosidases represent the final step of cellulose hydrolysis by converting cellobiose and soluble cellodextrins into fermentable glucose. This reaction is critical because cellobiose accumulation strongly inhibits exoglucanase activity and disrupts the synergistic action of cellulolytic enzymes during biomass saccharification. β-glucosidases cleave β-(1,4)-glycosidic bonds between glucose residues or glucose-substituted molecules, releasing free glucose units. In addition to their roles in lignocellulose degradation, these enzymes participate in diverse biological processes, including glycolipid degradation, cell wall metabolism, phytohormone activation, and plant–microbe interactions [66,67].

A major limitation of β-glucosidases in industrial biomass conversion is product inhibition caused by glucose accumulation. Consequently, considerable efforts have focused on engineering glucose-tolerant variants through rational mutagenesis, directed evolution, and computational protein design. For example, You et al. engineered CaBGL from *Caldicellulosiruptor* sp. F32 using a thermostable GFP-based high-throughput screening platform combined with structure-guided semi-rational design. The resulting M418T variant exhibited more than twofold enhanced catalytic activity in the presence of glucose and increased cellulose saccharification efficiency by 43.27% compared with the wild-type enzyme [68]. Similarly, computational stability-guided engineering using FireProt identified the Q302P mutation in a GH1 β-glucosidase, improving thermostability, substrate affinity, and glucose tolerance by modifying structural features associated with substrate binding and product release [69]. These studies demonstrate that engineering catalytic tunnel architecture, substrate-binding regions, and conformational flexibility can simultaneously improve enzyme activity and resistance to product inhibition.

Beyond individual enzyme optimization, structural biology and computational approaches are increasingly being integrated to accelerate β-glucosidase discovery and design. Machine learning-assisted analysis of genomic, transcriptomic, secretomic, and structural datasets provides opportunities to identify novel GH1 and GH3 β-glucosidases with de-

sirable catalytic properties. Candidate enzymes can subsequently be evaluated using molecular docking, molecular dynamics simulations, and structure-guided mutagenesis to identify residues involved in substrate recognition, glucose inhibition, thermal stability, and catalytic turnover. Importantly, integrating expression and secretion profiles enables the distinction between genes that are present in fungal genomes and those actively involved in lignocellulose degradation, providing valuable targets for promoter engineering and transcriptional regulation.

In filamentous fungi, balancing β-glucosidase production with endoglucanase and exoglucanase secretion remains essential for efficient cellulose conversion. Although *T. reesei* is widely exploited for industrial cellulase production, its relatively low β-glucosidase activity often results in incomplete cellobiose conversion and reduced saccharification efficiency. Therefore, supplementation with β-glucosidase-rich fungi such as *A. niger*, heterologous expression, and engineered fungal strains have been explored to improve cellulase synergy. Future developments integrating enzyme engineering, computational design, fungal regulatory-network reconstruction, and multi-enzyme cocktail optimization are expected to generate adaptive cellulase systems capable of efficient conversion of diverse lignocellulosic feedstocks into fermentable sugars.

3.2. Multi-Enzyme Cocktail

Due to the highly recalcitrant structure of lignocellulosic biomass, efficient enzymatic hydrolysis requires the synergistic action of multiple hydrolytic enzymes rather than a single biocatalyst. Cellulose degradation primarily involves the coordinated activities of cellobiohydrolases (CBHs), endoglucanases (EGs), and β-glucosidases (BGLs), which collectively hydrolyze crystalline and amorphous cellulose into fermentable sugars. CBHs initiate the progressive cleavage of cellulose chains from exposed chain ends, whereas EGs generate new chain ends through random cleavage of internal glycosidic bonds. Subsequently, BGLs convert cellobiose into glucose, reducing product inhibition and improving overall saccharification efficiency. However, complete biomass deconstruction requires additional accessory enzymes, including xylanases, mannanases, pectinases, esterases, and lignin-modifying enzymes, due to the complex interactions between cellulose, hemicellulose, and lignin within the plant cell wall.

Filamentous fungi are considered promising sources of multi-enzyme cocktails due to their natural ability to secrete a broad spectrum of extracellular hydrolytic enzymes. Unlike purified commercial enzymes containing only selected catalytic activities, fungal secretomes typically consist of diverse enzyme mixtures that collectively target different components of lignocellulosic biomass. For instance, a study demonstrated the potential of rice bran, a low-value agricultural residue, as an inducer for fungal enzyme production, resulting in the generation of enzyme cocktails containing cellulases, xylanases, glucosidases, galactosidases, and other accessory enzymes [70]. Such naturally derived enzyme mixtures may provide improved biomass hydrolysis efficiency because the enzymes function synergistically to overcome structural barriers within lignocellulosic substrates.

Among the investigated fungal isolates, *A. niger* var. *phoenicis* exhibited the highest production of hemicellulolytic enzymes, with xylanases accounting for approximately 39.2% of the secretome, followed by *A. terreus* (22.7%) and *Penicillium parvofructum* (5.8%) [70]. These findings corresponded with their measured xylanase activities, where *A. niger* var. *phoenicis* demonstrated the highest hydrolytic performance, followed by *A. terreus* and *P. parvofructum*. Nevertheless, enzyme abundance within fungal secretomes does not always directly correlate with overall biomass conversion efficiency. The effectiveness of an enzyme cocktail depends on multiple factors, including substrate accessibility, enzyme synergistic interactions, adsorption behavior, resistance to inhibitors

generated during pretreatment, and compatibility with the physicochemical properties of the target biomass. Therefore, enzyme cocktails developed for industrial applications should be optimized according to the specific composition and recalcitrance of the intended feedstock rather than relying solely on maximizing the number or concentration of enzymes produced.

The development of highly efficient enzyme catalysts remains one of the major challenges limiting the commercialization of second-generation bioethanol production. Although fungal enzyme cocktails provide a natural solution for biomass degradation, their catalytic performance can vary significantly depending on fungal species, cultivation conditions, and substrate composition. Consequently, identifying and improving enzymes with enhanced activity, stability, and substrate specificity has become an important research focus. Computational approaches have emerged as valuable complementary strategies for accelerating enzyme discovery and optimization by predicting structural characteristics, catalytic mechanisms, substrate interactions, and potential performance prior to experimental validation [53]. These approaches can reduce screening time and experimental costs while facilitating rational enzyme engineering.

Despite these advantages, computational methods also have limitations. Predictions are often dependent on the availability and accuracy of structural databases and may not fully capture the complexity of industrial hydrolysis conditions. Factors such as heterogeneous biomass composition, non-productive enzyme adsorption onto lignin, synergistic interactions among multiple enzymes, and inhibition by pretreatment-derived compounds remain difficult to predict accurately using computational models alone. Therefore, computational screening should be integrated with experimental approaches, including biochemical characterization and pilot-scale validation, to ensure the practical applicability of identified enzyme candidates.

Recent advances have demonstrated the potential of highly diversified enzyme formulations for enhanced biomass conversion. For example, a multiple hydrolytic enzyme cocktail consisting of 19 concentrated enzyme components was developed, exhibiting activities of 150–250 IU/mL CMCase, 30–40 IU/mL FPase, 25–35 IU/mL Avicelase, 30–40 IU/mL β-glucosidase, 135–145 IU/mL cellobiase, 160–175 IU/mL salicinase, 800–900 IU/mL xylanase, 50–70 IU/mL xylosidase, 260–275 IU/mL mannanase, 25–35 IU/mL mannosidase, 25–35 IU/mL pectin lyase, 25–35 IU/mL polygalacturonase, 12,500–15,000 U/mL α-amylase, 50–75 IU/mL pullulanase, 400–500 IU/mL glucoamylase, 140–165 IU/mL α-glucosidase, 2100–2300 U/mL protease, 190–210 U/mL lipase, and 190–210 U/mL alginate lyase [71,72]. Such comprehensive enzyme systems highlight the potential of combining multiple catalytic activities to achieve extensive degradation of complex biomass structures.

However, the industrial application of highly complex enzyme cocktails remains challenging. The production of numerous enzyme components requires additional fermentation, recovery, and formulation processes, which may increase manufacturing costs and reduce economic feasibility. Furthermore, increasing the number of enzymes within a cocktail does not necessarily result in proportional improvements in sugar release, as some enzymes may exhibit overlapping functions or limited accessibility to their target substrates. Therefore, future research should focus on developing optimized enzyme formulations containing essential catalytic components with high synergistic efficiency, rather than simply increasing enzyme diversity. Advances in fungal strain improvement, secretome engineering, recombinant enzyme production, and computational-guided cocktail design may provide promising strategies for achieving cost-effective and efficient enzymatic hydrolysis for industrial-scale second-generation bioethanol production.

3.3. Challenges Associated with Lignocellulose Recalcitrance and Pretreatment Dependence

Lignocellulosic biomass has emerged as a promising renewable feedstock for second-generation (2G) bioethanol production due to its abundance, low cost, and potential contribution to sustainable bioenergy generation. However, efficient fractionation and complete valorization of its major components, including cellulose, hemicellulose, and lignin, remain challenging due to the highly complex and recalcitrant structure of lignocellulosic biomass.

Pretreatment methods are designed to disrupt the complex physical and chemical structure of lignocellulosic biomass, thereby improving cellulose accessibility for subsequent enzymatic hydrolysis. In addition to improving enzyme accessibility, certain pretreatment approaches can partially separate lignin, hemicellulose, and cellulose fractions, facilitating their subsequent conversion into valuable products. These processes enhance biomass deconstruction by modifying structural characteristics associated with recalcitrance, including biomass porosity, cellulose degree of polymerization (DP), cellulose crystallinity, and lignin composition and content.

However, conventional pretreatment methods, particularly severe chemical and thermal processes, may generate inhibitory compounds such as furfural, hydroxymethylfurfural (HMF), and phenolic compounds through carbohydrate and lignin degradation. These compounds can inhibit fungal growth, reduce ligninolytic and cellulolytic enzyme production, and negatively affect subsequent ethanol fermentation. To overcome this bottleneck, future strategies should focus on developing mild and selective pretreatment approaches that maximize lignin removal and cellulose accessibility while minimizing inhibitor formation.

Cost-Effective Pretreatment

Beyond technical challenges, the economic feasibility of pretreatment remains a major bottleneck in large-scale 2G ethanol production. Pretreatment accounts for approximately 18% of the total production cost of cellulosic ethanol [33]. Among physical pretreatment approaches, energy consumption varies considerably depending on the method applied. Mechanical milling requires substantially higher energy input (4.0–12.5 MJ/kg biomass), whereas steam explosion requires significantly lower energy input (0.2–0.6 MJ/kg biomass). The energy required to produce milled biomass with a particle size of 0.5 mm was reported to be nearly 22 times higher than that required for steam explosion treatment at 2.8 MPa pressure [73]. Although steam explosion and chemical pretreatments improve cellulose and hemicellulose accessibility by disrupting lignocellulosic structures, their industrial application remains limited due to high energy requirements and the formation of fermentation inhibitors, such as furfural and HMF [74]. Therefore, current research on 2G ethanol production focuses on developing cost-effective pretreatment technologies, optimizing existing processes, and exploring alternative biomass feedstocks, particularly agricultural and industrial residues.

3.4. Molecular Stress-Response Pathways Underlying Inhibitor Tolerance

Transcriptome-level evidence in the filamentous fungus *Aspergillus oryzae* RIB40 shows that lignocellulosic inhibitors (furfural, HMF, ferulic acid, vanillin, levulinic acid) converge on a reactive-oxygen-species (ROS)–dominated stress program, with strong early repression of cell-cycle, ribosome, and ER-protein-folding functions and coordinated upregulation of NAD(P)H-dependent oxidoreductases and efflux systems (ABC and MFS transporters) that limit intracellular toxin burden [75]. This architecture mirrors broader fungal inhibitor-tolerance logic: aldehydic furans and phenolics deplete NAD(P)H, inhibit glycolytic/TCA enzymes, and damage DNA and membranes, so cells reroute carbon through the pentose phosphate pathway to regenerate NADPH for glutathione/thioredoxin recycling and for

aldehyde reductases that convert furfural/HMF to less toxic alcohols. In filamentous taxa, MAPK modules (HOG/CWI) and bZIP/AP-1-like regulators integrate osmotic, oxidative, and cell-wall cues to drive expression of catalases, peroxidases, glycerol biosynthesis, chaperones, and transporters, thereby preserving proteostasis and membrane integrity; similar transcriptional rewiring has been reported in *Trichoderma* and other ascomycetes under chemical and osmotic stress. Together, these data position ROS detoxification, NADPH-linked aldehyde reduction, efflux, and MAPK-mediated transcriptional reprogramming as the core, generalizable determinants of inhibitor tolerance in filamentous fungi used for 2G ethanol processes.

Beyond canonical oxidative-stress and detoxification circuits, the architecture of filamentation-linked signaling itself is environmentally contingent, a principle highlighted by [76], who showed that distinct ecological inducers (e.g., non-preferred carbon sources, ethanol, pectin) rewire the yeast filamentous growth MAPK (fMAPK) pathway and its crosstalk with Ras–cAMP–PKA, rendering some sensors and kinases dispensable under specific conditions while making others essential. In the context of 2G ethanol production, where fungi encounter mixed inhibitors (furans, weak acids, phenolics) alongside high ethanol and carbon-source shifts, this implies that the relative contribution of MAPK modules (HOG/CWI/fMAPK-like), Ras–PKA, and downstream transcriptional programs to tolerance may change with hydrolysate composition and process stage. Practically, this supports an environment-aware engineering strategy: rather than assuming a fixed "core" set of tolerance genes, strain design should consider condition-dependent pathway usage, combinatorial inducer effects (e.g., ethanol plus carbon stress), and potential redundancy among sensors, adaptors, and kinases that only emerge under industrially relevant, multi-stress conditions.

Collectively, these interconnected stress-response pathways enable filamentous fungi to maintain cellular homeostasis, preserve metabolic activity, and sustain ethanol fermentation under the complex inhibitory conditions characteristic of lignocellulosic hydrolysates. Understanding these regulatory networks provides valuable targets for metabolic engineering aimed at improving fungal robustness and industrial-scale bioethanol production.

4. Qualities of Filamentous Fungi and Their Advantages in Lignocellulosic Biorefinery

The economic viability of biorefineries can be improved by relying on low-cost feedstocks such as lignocellulosic residues and by integrating multiple product streams to enhance revenue generation [23]. Among the many requirements for the success of lignocellulosic biorefineries, efficient enzymatic hydrolysis is critical; however, the high cost of cellulase production continues to be a major obstacle to achieving commercial-scale application [77].

Fungi exist in a wide range of forms, from microfungi like molds and yeasts to macrofungi such as mushrooms and truffles. Larger fungi are commonly consumed as food or nutritional supplements, while microfungi, including *Aspergillus*, *Penicillium*, and *Saccharomyces*, are widely utilized for the synthesis of enzymes and valuable metabolites [78]. Yeast has been exploited for centuries in the production of alcoholic beverages and baked goods. Compared to other microorganisms, yeast offers significant advantages, including a shorter generation time, no requirement for arable land, and the ability to grow on a broad spectrum of renewable substrates derived from agro-industrial by-products and municipal solid waste [79,80]. Ethanol fermentation from glucose is traditionally carried out with pure cultures of selected species such as *S. cerevisiae*. Engineered strains of *S. cerevisiae* have been developed to metabolize xylose, and some have even been modified

to ferment cellobiose in order to minimize glucose repression during biomass conversion. However, their industrial application remains constrained, particularly due to drawbacks such as extended lag phases [81]. In contrast, the use of cellulolytic filamentous fungi to ferment biomass-derived sugars presents notable advantages, making them strong candidates for advancing bioethanol production in biorefineries.

Filamentous fungi are particularly attractive for biorefinery applications because of their adaptability, resilience, and efficiency in breaking down complex organic substrates into simpler molecules through their hydrolytic enzyme systems [82]. They encompass diverse groups such as zygomycetes, basidiomycetes, and ascomycetes, with genera including *Aspergillus*, *Rhizopus*, *Trichoderma*, *Penicillium*, *Fusarium*, and *Neurospora* being of great industrial importance [24]. These fungi extend their mycelia into surrounding substrates, capturing and incorporating organic matter into their hyphal networks to form composite structures. In mushroom-forming species such as *Ganoderma lucidum* and *Pleurotus ostreatus*, glucan- and chitin-rich hyphae bind cellulose- and lignin-containing plant materials, resulting in a strong interconnected matrix [83]. Through the action of their enzyme systems, fermentable sugars are released from holocellulose, which consists of cellulose and hemicellulose, and these sugars are subsequently fermented into ethanol. One of the major challenges in producing 2G ethanol is the high cost of saccharification, which contributes nearly half of the total production expense. Addressing this issue has prompted an increased research focus on lignocellulolytic fungi, as these organisms possess the enzymatic machinery necessary to efficiently degrade lignocellulosic biomass into fermentable sugars that can be converted into bioenergy [21].

4.1. Fungal Strains Utilized for Bioethanol Production

Enzymatic hydrolysis is considered one of the most expensive steps in bioethanol production, significantly contributing to overall costs and limiting large-scale implementation. One strategy to address this challenge is the on-site production of enzymes, which reduces dependence on costly commercial preparations [48]. Several filamentous fungi have shown the capacity to secrete hydrolytic enzymes and, in certain cases, directly ferment lignocellulosic substrates without the need for added enzymes. For example, ascomycetes, such as *Fusarium*, *Neurospora*, and *Monilia*, along with basidiomycetes, including *Phlebia* and *Trametes*, are able to produce ethanol directly from cellulose and chemically pretreated biomass [84].

Among these fungi, *T. reesei* stands out as an efficient cellulase producer, supporting its widespread use in converting lignocellulosic biomass into ethanol and other value-added products such as xylitol and carboxylic acids. *A. niger* is extensively utilized for the production of β-glucosidase and citric acid, while *Fusarium oxysporum* demonstrates the ability to both produce cellulases and ferment the released sugars into ethanol [85]. Table 1 provides a comprehensive overview of filamentous fungi with high enzymatic activity for saccharification, highlighting their potential to improve biomass hydrolysis efficiency and reduce production costs.

Table 1. Representative filamentous fungi exhibiting high reported enzymatic activities for lignocellulose saccharification under optimized fermentation conditions.

Species	Main Substrate	Bioprocess	Enzymatic Activity			References
			CMCase	Fpase/FPU	β-Glucosidase	
Aspergillus niger DM	Defatted rice bran	Solid-state fermentation (SSF)	146.44 ± 1.22 U/g	27.23 ± 2.76 U/g	97.51 ± 4.53 U/g	[86]
Aspergillus fumigatus SCBM6	Sugarcane bagasse	Solid-state fermentation (SSF)	82.70 U/g	9.48 U/g	50.36 U/g	[87]
Rhizopus oryzae UC2	Raw oil palm frond leaves (OPFL)	Solid-state fermentation (SSF)	94.68 U/g	25.46 U/g	145.47 U/g	[88]
Fusarium oxysporum F3	Wheat bran	Solid-state cultivation (SSC)	65.2 U/g	1.5 U/g	27.4 U/g	[48]
Neurospora crassa NRRL-2332	Canola meal	Solid-state fermentation (SSF)	2.47 ± 0.23 U/g	1.44 ± 0.20 U/g	190.84 ± 12.24 U/g	[89]
Trichoderma reesei	Canola meal	Solid-state fermentation (SSF)	1.35 ± 0.07 U/g	0.17 ± 0.08 U/g	440.02 ± 6.18 U/g	[89]
Trametes versicolor 561	Sugarcane bagasse	Solid-state fermentation (SSF)	56.51 U/g	9.45 U/g	80.31 U/g	[87]
Pleurotus ostreatus PL06	Sugarcane bagasse	Solid-state fermentation (SSF)	19.90 U/g	6.68 U/g	1.60 U/g	[87]

Note that the enzymatic activities summarized in Table 1 were obtained from individual studies using different fermentation substrates, solid-state cultivation parameters, and analytical methodologies. Therefore, these values should not be considered directly comparable across fungal species. Instead, the table highlights representative examples of filamentous fungi exhibiting strong cellulolytic potential under their respective optimized fermentation conditions. Comparative evaluation of fungal enzyme production requires standardized benchmarking using identical substrates, cultivation parameters, and enzyme assays.

4.1.1. *Aspergillus niger*

Fungi are recognized as the principal producers of cellulases, and *A. niger* has been extensively utilized in industry because of its efficiency in secreting cellulolytic enzymes [90]. This mold is widely applied in different fermentation processes to produce enzymes such as pectinase, lactase, cellulase, and amylase, and it is also capable of metabolizing lignocellulose-derived compounds, including acetic acid, furanic aldehydes, and phenolic compounds, which usually act as inhibitors during ethanol fermentation [91]. Furthermore, *A. niger* serves as a well-established model microorganism for the expression of industrial enzymes, and several enzymes have been successfully expressed in this species, including lignin peroxidase, ferulic esterase, and manganese peroxidase [92]. It is also considered an important producer of β-glucanase [93]. One of the most widely used commercial β-glucosidase preparations, Novozymes SP 188, produced by Novo Nordisk A/S in Denmark, originates from *A. niger* [94].

4.1.2. *Aspergillus oryzae*

Aspergillus oryzae, commonly referred to as "Koji mold," is a vital fungus used in the fermentation of various traditional East Asian foods, including rice wine, soy sauce, rice vinegar, black rice vinegar, and fermented soybean paste [95]. Over an extended adaptation period, this species has developed a strong system for protein secretion and post-translational modification, enabling the production of enzymes such as glucoamylase, cellulase, and protease [96]. It also has the ability to secrete large quantities of amylase, which hydrolyzes starch into glucose. The glucose released can be further metabolized into smaller sugars via glycolysis and eventually converted to ethanol under anaerobic fermentation conditions [97].

4.1.3. *Fusarium oxysporum*

The genus *Fusarium* includes many filamentous fungi that are known as producers of mycotoxins, plant pathogens of agricultural significance, and opportunistic human pathogens. They are cosmopolitan fungi commonly found in water, soil, and plant-associated habitats [98]. Among them, *F. oxysporum* is one of the few filamentous fungi that can ferment ethanol directly from lignocellulosic biomass. It possesses the essential enzymatic machinery required to break down lignocellulose into monosaccharides, which are subsequently fermented to ethanol under anaerobic or microaerobic conditions [99]. This fungus is known for secreting large amounts of proteins, thereby increasing productivity in biosynthetic processes, and it is capable of both saccharification and fermentation, making it highly suitable for bioethanol production [100]. In addition, it can penetrate the lignin barrier of plants and produce a wide range of enzymes, including cellulase, xylanase, β-glucosidase, pectinase, laccase, and lignin peroxidase, which strengthens its potential as a microbial agent in consolidated bioprocessing systems [101]. *F. oxysporum* is also tolerant to sugars and inhibitors such as acetate. During ethanol fermentation, acetate is typically produced as a byproduct, but this fungus is able to convert acetate into ethanol [102,103]. Because of its many metabolic advantages, *F. oxysporum* isolates have been tested on different biomass types and demonstrated high enzymatic activity, effective conversion of sugars, the ability to ferment both pentoses and hexoses derived from biomass, and a strong tolerance to ethanol [104]. A locally isolated strain of *F. oxysporum* produced maximum bioethanol from *Ficus cunia* fruits using consolidated bioprocessing at 5% substrate concentration and pH six, achieving yields of 3.54 g/L at 28 °C and 3.88 g/L at 32 °C, with only minor effects from temperature changes [99].

4.1.4. *Neurospora crassa*

Neurospora crassa is a rapidly growing fungus that naturally degrades cellulose and has a genome encoding approximately twice the number of cellulases found in *Trichoderma reesei*. It can efficiently utilize a range of carbon sources, including major lignocellulosic sugars such as glucose and xylose, making it highly suitable for research on consolidated bioprocessing or CBP [105]. This saprophytic fungus is well known for its secretion of plant cell wall-degrading enzymes and for its strong cellulase production capacity. It produces large quantities of the three key enzymes involved in cellulose breakdown, with cellobiohydrolase I (CBHI) being the most abundant extracellular protein acting on cellulose. Despite being used for more than a century as a model organism, its industrial potential has not yet been fully explored [106]. *N. crassa* is widely recognized as a valuable model system for biomolecular studies and offers advantages for genetic manipulation [107]. The fungus secretes a mixture of cellulases capable of breaking down cellulose and other polysaccharides in plant cell walls [108]. Its β-glucosidase system is well characterized in biotechnology, consisting of seven genes from GH families 1 and 3, with GH1-1 being an intracellular enzyme, GH3-4 occurring mainly in the culture medium, and GH3-3 predominantly associated with the cell wall, all contributing significantly to β-glucosidase activity [109]. Research has shown that *N. crassa* is able to detect the concentration of cellobiose in its surroundings, transport this compound into the cell through cellodextrin transporters on the plasma membrane, and subsequently activate the cellulase transcription factor to enhance cellulolytic enzyme expression [110].

According to [106], *N. crassa* exhibits higher extracellular protein production than *Trichoderma reesei*, which points to its industrial potential. The deletion of the pla-7 gene has been found to improve biomass yield and hyphal morphology, providing solutions to challenges faced in bioreactor applications. Moreover, *N. crassa* secretes a diverse set of enzymes for lignocellulose degradation, and several carbohydrate-active enzyme or CAZyme genes, including 14 AA9 LPMO genes, are strongly induced during growth on crop straw residues [111].

4.1.5. *Trichoderma reesei*

The genus *Trichoderma* is widely recognized for its agricultural and industrial importance. It is extensively applied in agriculture as a biological control agent against plant diseases [112]. Within this genus, *T. reesei* is commercially employed for the production of cellulolytic and hemicellulolytic enzymes, making it a key species in second-generation biofuel development [113]. Among the enzymes produced by *Trichoderma*, cellulolytic enzymes, such as cellobiohydrolases, endoglucanases, and β-glucosidases, along with lignocellulolytic enzymes, including xylanases and β-xylosidases, are the most widely studied because of their central role in biofuel production from lignocellulosic biomass [114]. *T. reesei* has been one of the most investigated fungi for cellulase synthesis and is considered a model species for understanding cellulolytic fungal systems [115]. The strains of *T. reesei* mainly produce two cellobiohydrolases (CBHI and CBHII), two endoglucanases (EGI and EGII), β-glucosidase I, xylanases, and a variety of auxiliary proteins [116].

One of the limitations of *T. reesei* is its lack of an effective lignin-degrading enzyme system, which reduces its efficiency in generating fermentable sugars and results in higher pre-treatment costs for biofuel production [117]. To overcome this drawback, several strategies have been adopted to improve the enzyme mixtures, including random mutagenesis and genetic engineering approaches, which have successfully enhanced the enzyme expression of *T. reesei* [118].

4.1.6. *Penicillium chrysogenum/rubens*

Species of the genus *Penicillium* are known producers of essential enzymes such as cellulases, hemicellulases, including xylanases and arabinofuranosidases, pectinases, and β-glucosidases, which contribute to the breakdown of plant cell walls into simple sugars for industrial uses [119]. *Penicillium chrysogenum* (currently reidentified as *Penicillium rubens*), originally studied for its antibiotic properties, has recently gained attention as a producer of cellulases, xylanases, and pectinases. This species has been described as an efficient producer of xylanases rather than cellulases, releasing a wide variety of hemicellulases such as endoxylanases, β-xylosidases, α-galactosidases, α-arabinofuranosidases, β-mannosidases, and carboxylesterases when grown on wheat bran combined with microcrystalline cellulose as a carbon source [120,121]. According to [122], *P. rubens* produces cellulase at 0.71 ± 0.09 U/mL and β-glucosidase at 4.35 ± 0.38 U/mL, making it effective for cell wall hydrolysis and polydatin deglycosylation. In addition, a locally isolated strain of *P. chrysogenum* (A3 DSM105774) was successfully applied in the valorization of wheat straw, a byproduct of lignocellulose processing, achieving a nearly ninefold increase in xylanase production after optimization strategies compared with its initial yield [123].

4.2. Influence of Fungal Morphology on Fermentation Performance and Industrial Scale-Up

Beyond enzymatic capability, fungal morphology represents a critical factor influencing the industrial performance of filamentous fungi in lignocellulosic bioethanol production. Filamentous fungi exhibit diverse growth forms, ranging from dispersed mycelial networks to compact pellet structures, which are determined by both genetic traits and environmental conditions, including pH, agitation, aeration, osmotic pressure, and inoculum size. Pellet formation can be advantageous in industrial fermentation by reducing broth viscosity, improving mixing efficiency, and facilitating downstream processing. However, excessive pellet development may create diffusion limitations that restrict oxygen and nutrient penetration into the inner regions of fungal aggregates, thereby reducing metabolic activity and overall productivity [124].

The industrial potential of filamentous fungi is therefore influenced not only by biomass yield, cell wall properties, and enzyme production capacity, but also by their growth morphology and hyphal organization [125]. The rapid extension and branching of fungal hyphae facilitate efficient substrate colonization, allowing fungi to exploit available nutrients while limiting the establishment of competing microorganisms. In addition, the secretion of extracellular hydrolytic enzymes enables the efficient breakdown of complex lignocellulosic polymers into accessible carbon sources, further enhancing fungal competitiveness and substrate utilization. Consequently, improved nutrient acquisition by filamentous fungi may reduce resource availability for other microorganisms, contributing to their ecological dominance during biomass degradation processes [59].

Since hyphal growth patterns and morphological characteristics directly regulate mass transfer, enzyme secretion, substrate accessibility, and stress tolerance, they strongly influence fermentation performance, including production efficiency, product quality, and adaptability under varying cultivation conditions. Therefore, a comprehensive understanding and precise regulation of fungal morphogenesis are essential for optimizing fermentation processes and improving the scalability of filamentous fungi-based bioethanol production systems [126].

5. Co-Culture Strategies with Yeasts

Saccharomyces cerevisiae is regarded as one of the most reliable industrial microorganisms for ethanol production because of its resilience and high efficiency. Large-scale bioethanol production mainly relies on this yeast due to its capacity to convert sugars from

feedstock into ethanol rapidly and almost completely [16]. Despite these advantages, the build-up of ethanol during fermentation reduces the performance of *S. cerevisiae*, as high ethanol concentrations exert toxic effects that lower cell viability, trigger stress responses, and alter both gene expression and metabolic pathways [127].

To address these challenges, co-cultivation of yeasts has become an attractive strategy, especially for lignocellulosic biomass fermentation. These consortia enable the simultaneous use of pentose and hexose sugars, overcoming the limited substrate spectrum of *S. cerevisiae* and thereby improving sugar utilization and ethanol production [128]. In natural ecosystems, microorganisms live in dynamic communities where interactions such as competition, mutualism, commensalism, and antagonism play a vital role in regulating decomposition processes and maintaining ecological balance [129]. Co-culture systems reflect these natural interactions and can be applied in biotechnology because of their adaptability, resilience, and ability to accomplish complex biochemical functions. Nevertheless, their effectiveness is highly dependent on species compatibility, as even microorganisms from closely related groups may behave differently, which means each pairing must be carefully evaluated [130]. In some cases, incompatibility leads to competition or antagonism, reducing process efficiency. This makes it important to monitor population dynamics using methods that range from conventional approaches, such as microscopy and colony counts, to more advanced tools, including quantitative PCR, flow cytometry with fluorescent markers, and fluorescence in situ hybridization [131].

5.1. Yeast Immobilization

Yeast immobilization involves enclosing active and intact cells within a defined environment in such a way that their biological activity is preserved. This method has been shown to improve tolerance against inhibitors derived from lignocellulosic biomass and to enhance the simultaneous use of different sugars [132]. A notable example is the co-culture of *S. cerevisiae* with *Penicillium chrysogenum* through a system known as yeast biocapsules. In this arrangement, both microorganisms adhere to each other to form spherical structures, which limit fungal growth while allowing immobilized yeast cells to proliferate continuously [133]. Yeast biocapsules have been reported to produce bioethanol at concentrations of 95 to 98 g/L, achieving yields between 0.49 and 0.54 g ethanol per gram of glucose. Comparable performance has also been demonstrated with enzymatic hydrolysates of palm-derived lignocellulosic residues, yielding about 0.49 ± 0.17 g/g. This self-organized encapsulation of microorganisms suggests strong potential as a reusable biocatalyst system with high retention of active yeast cells [134].

Applications of yeast biocapsules extend beyond bioethanol production, as they have been employed in producing alcoholic beverages such as white wine, sparkling wine, natural sweet wine, and long-aged sparkling wines. However, similar to other immobilization techniques, one of their limitations is the gradual leakage of yeast cells from the supporting structure [135].

5.2. Co-Culturing Enzymes

Balanced enzyme production remains a major bottleneck limiting the industrial competitiveness of filamentous fungi for second-generation bioethanol production. Individual fungal strains rarely secrete all cellulolytic and hemicellulolytic enzymes at levels sufficient for efficient lignocellulose deconstruction. Consequently, co-culture strategies have emerged as an effective approach to exploit the complementary metabolic capabilities of different microorganisms, enabling the production of more balanced and synergistic enzyme systems [66]. Compared with monocultures, fungal consortia can enhance enzyme secretion, improve saccharification efficiency, facilitate lignin degradation, and promote more

complete utilization of lignocellulosic substrates. Additional benefits include improved biomass conversion, efficient utilization of inexpensive feedstocks, alleviation of nutrient limitations, enhanced resistance to contamination, and greater process flexibility [136].

Among filamentous fungi, *T. reesei* remains the benchmark producer of cellulases and is widely employed in co-culture systems because of its high cellulolytic capacity. However, its relatively low β-glucosidase production often limits complete cellulose hydrolysis. Co-cultivation with complementary microorganisms capable of producing β-glucosidase or other accessory enzymes helps overcome this limitation, thereby improving the overall efficiency of lignocellulose saccharification [118]. These findings demonstrate that enzyme synergy, rather than the performance of a single strain, is often the key determinant of efficient biomass conversion.

Several studies have demonstrated the potential of co-culture systems for bioethanol production. For example, *Saccharomyces cerevisiae* co-cultured with *Trichoderma harzianum* and supplemented with cellulase and β-glucosidase produced an ethanol concentration of 11.76 g/L and a yield of 0.29 g/g from pretreated empty fruit bunch (EFB) after 72 h of fermentation [137]. Similarly, a mixed crude culture of *T. reesei* and *S. cerevisiae* converted Napier grass into ethanol, producing 25.02 g/L without external sugar supplementation and up to 33.24 g/L following sugar addition, while simultaneously reducing process complexity and capital investment [138]. Collectively, these studies demonstrate that co-culture systems can improve enzyme complementarity, biomass utilization, and ethanol production compared with conventional monoculture processes.

Despite these promising laboratory-scale results, the industrial application of fungal co-cultures remains challenging. The stability of microbial consortia is strongly influenced by interactions that may be synergistic, neutral, or antagonistic, making fermentation outcomes difficult to predict and reproduce [129,139]. Differences in growth kinetics, substrate preferences, oxygen requirements, and metabolite production can result in competitive exclusion, population imbalance, and reduced ethanol productivity. Furthermore, maintaining a stable microbial community over prolonged industrial fermentations increases the complexity of contamination control, process monitoring, and quality assurance [129]. Most reported co-culture systems have also been evaluated under controlled laboratory conditions, with limited validation at pilot or commercial scales.

Overall, current evidence suggests that fungal–yeast co-culture represents a promising strategy for overcoming the limitations of individual strains by generating complementary enzyme systems and improving biomass conversion efficiency. However, its successful industrial implementation will require a deeper understanding of microbial interactions, the development of robust and stable microbial consortia, and advanced bioprocess control strategies capable of maintaining reproducible performance during large-scale bioethanol production.

5.2.1. Metabolic Crosstalk and Resource Partitioning in Fungal–Yeast Consortia

Metabolic crosstalk is a central determinant of the performance, stability, and product spectrum of fungal–yeast consortia developed for second-generation (2G) ethanol production. Rather than functioning as a simple mixture of independent microorganisms, a consortium operates as an interconnected metabolic network in which one member modifies the substrate or extracellular environment, while another consumes the resulting intermediates and directs carbon flux toward ethanol production. The cross-kingdom metabolic interactions can involve nutrient exchange, organic acids, redox-active metabolites, signaling molecules and secondary metabolites; these concepts provide a useful framework for interpreting fungal–yeast consortia used in bioprocessing. Such exchanges can reduce

the metabolic burden on individual members, expand substrate utilization capacity, and improve community-level productivity compared with monoculture systems [140].

In fungal–yeast consortia, metabolic specialization enables functional division of labor during lignocellulose conversion. Filamentous fungi primarily contribute through extracellular secretion of cellulases, hemicellulases, and accessory enzymes that depolymerize complex lignocellulosic structures into soluble sugars, including glucose, cellobiose, xylose, and arabinose. These hydrolysis products subsequently serve as substrates for fermentative yeasts, which efficiently channel carbon toward ethanol production. Continuous removal of soluble sugars by yeast may reduce product accumulation and alleviate feedback inhibition, thereby supporting sustained fungal enzyme production during prolonged fermentation. This complementary relationship allows fungi to focus on biomass deconstruction while yeasts function as efficient sugar sinks, improving the coupling between hydrolysis and fermentation.

The partitioning of sugar metabolism is particularly important because lignocellulosic hydrolysates contain heterogeneous mixtures of hexose and pentose sugars. Conventional *S. cerevisiae* strains efficiently ferment glucose but generally possess limited native capacity for pentose utilization. Consequently, preferential glucose uptake can suppress xylose assimilation through carbon catabolite repression, resulting in incomplete carbon recovery from lignocellulosic substrates. In fungal–yeast systems, this limitation can be addressed by combining fungal hydrolytic capabilities with yeast populations possessing complementary sugar utilization capacity, including pentose-fermenting or metabolically engineered strains. Experimental studies on engineered yeast consortia have demonstrated that consortium composition and the timing of population development strongly influence mixed-sugar fermentation, indicating that effective division of labor depends not only on strain selection but also on temporal coordination between microbial partners [141].

A direct example of fungal–yeast resource partitioning was demonstrated by [25], who employed *T. reesei* Rut-C30 and *S. cerevisiae* in an integrated continuous bioethanol production system. In this configuration, *T. reesei* supplied cellulolytic and hemicellulolytic activities to release fermentable sugars from lignocellulosic substrates, whereas *S. cerevisiae* converted the liberated sugars into ethanol. The system operated continuously for more than 60 days and achieved approximately 60–70% conversion of the relevant carbohydrate substrates, illustrating stable coupling of fungal saccharification and yeast fermentation. These findings demonstrate how metabolic functions can be distributed between fungal and yeast partners to improve substrate conversion while reducing the limitations associated with single-organism platforms. However, the efficiency of such systems depends on maintaining a balance between fungal enzyme production, sugar release, and yeast fermentation capacity.

The exchange of metabolites between consortium members may be directly beneficial or conditionally beneficial depending on environmental conditions and population dynamics. During lignocellulose conversion, fungal degradation can generate soluble sugars, oligosaccharides, organic acids, and other metabolites that influence the growth and metabolism of partner microorganisms. For example, co-cultivation of anaerobic fungi with *Clostridium acetobutylicum* demonstrated that fungal degradation of lignocellulosic biomass released glucose, xylose, acetate, formate, ethanol, and lactate, enabling metabolic cross-feeding and subsequent production of butyrate and butanol [142]. Although this example involves a bacterial rather than a yeast partner, it demonstrates how fungal degradation can generate metabolites that support cross-feeding in lignocellulose-degrading communities. Yeasts can also contribute to hydrolysate detoxification by reducing furfural and HMF to less toxic derivatives through NAD(P)H-dependent reactions, although the extent to which this activity benefits a fungal partner is dependent on the consortium

and process conditions [143]. Therefore, fungal–yeast consortia can potentially integrate multiple functions, including polymer degradation, inhibitor detoxification, and sugar fermentation, within a single bioprocess.

Nevertheless, metabolic cooperation does not always result in synergistic outcomes. Competition for essential resources, including carbon sources, nitrogen, oxygen, and micronutrients, can alter population dynamics and compromise consortium stability. Rapid yeast proliferation may deplete available soluble sugars before sufficient fungal enzyme production occurs, whereas excessive fungal growth may increase broth viscosity, reduce oxygen transfer, and negatively affect yeast fermentation performance. Furthermore, accumulation of ethanol, organic acids, or fungal secondary metabolites may create selective pressures that favor one population over another, leading to community imbalance and reduced productivity. Therefore, successful fungal–yeast consortia require precise regulation of inoculation ratios, substrate availability, oxygen supply, and cultivation conditions to maintain a stable interaction between hydrolytic and fermentative partners.

Overall, fungal–yeast consortia represent a promising strategy for improving 2G ethanol production by integrating complementary metabolic functions. Filamentous fungi provide the enzymatic machinery required for lignocellulose deconstruction, while yeasts act as efficient fermentative partners that convert released carbohydrates into ethanol. This division of labor can reduce metabolic burden, enhance carbon recovery, and improve substrate utilization compared with monoculture systems. However, achieving industrially relevant performance requires a comprehensive understanding of interspecies interactions, including metabolite exchange, pathway competition, population dynamics, and resource allocation. Future development of robust fungal–yeast platforms will depend on maintaining a dynamic balance between hydrolysis, sugar uptake, fermentation, and population stability through the integration of metabolic engineering, microbial ecology, and advanced bioprocess control strategies to achieve reproducible performance at industrial scale [131].

5.2.2. Overcoming Metabolic Limitations

While fungal–yeast consortia provide complementary metabolic functions, their industrial implementation remains constrained by physiological limitations, environmental stresses, and scale-up challenges. Overcoming these limitations requires integrated strategies involving metabolic engineering, reactor design, and process optimization. The effectiveness of a consortium depends not only on the presence of complementary metabolic functions but also on the ability of individual members to withstand inhibitory compounds, maintain stable activity, and sustain productivity under industrial fermentation conditions.

Fungal partners such as *A. niger* are attractive candidates due to their strong lignocellulolytic capability and ability to tolerate relatively high organic loading and certain lignocellulose-derived inhibitors. However, fungal performance may still be affected by pretreatment-derived compounds, including furan derivatives, weak acids, and phenolic compounds, which can inhibit enzyme secretion, cellular metabolism, and overall hydrolytic efficiency. Furthermore, although in situ enzyme production provides an advantage by reducing enzyme supplementation costs, maintaining consistent enzyme expression and fungal activity during prolonged industrial fermentation remains a significant challenge. Advanced process strategies, including membrane-aerated bioreactors, immobilization carriers, and spatial niche engineering, have been explored to facilitate the coexistence of aerobic fungi with anaerobic or microaerophilic bacteria by creating suitable microenvironments [59]. Nevertheless, the scalability and economic feasibility of these approaches require further validation under industrial operating conditions. Moreover, the diverse morphologies of filamentous fungi introduce important scale-up challenges. Dispersed mycelial growth can increase broth viscosity, impair mixing and mass transfer, and produce

heterogeneous nutrient distributions, whereas dense pellets may develop internal oxygen and nutrient limitations. These morphology-dependent effects can negatively influence reactor performance and fermentation efficiency [144].

Beyond process optimization, metabolic engineering provides an additional strategy to enhance the performance of microbial partners by improving traits that are insufficient in native strains. An ideal lignocellulose-converting microorganism should exhibit efficient substrate uptake, broad sugar utilization capacity, tolerance toward pretreatment-derived inhibitors, high metabolic flux toward desired products, and resistance to product toxicity. However, naturally occurring microorganisms rarely possess all these characteristics simultaneously. Therefore, engineering strategies have focused on expanding substrate utilization capacity, reducing carbon catabolite repression to enable co-utilization of glucose and pentoses, improving inhibitor tolerance through adaptive evolution or global transcription machinery engineering, and redirecting metabolic pathways toward enhanced production of ethanol, butanol, and other advanced biofuels [145].

For filamentous fungi, metabolic engineering offers opportunities to improve enzyme secretion capacity, regulate carbon-responsive pathways, and enhance tolerance toward lignocellulose-derived stress factors. However, engineering fungal systems remains challenging compared with conventional bacterial or yeast hosts due to their complex regulatory networks, multicellular growth patterns, and difficulties in achieving predictable and stable genetic modifications. Furthermore, improvements at the genetic level do not always translate directly into higher industrial productivity because biomass heterogeneity, enzyme accessibility, inhibitor formation, and reactor conditions continue to influence overall process performance.

Therefore, overcoming metabolic limitations in lignocellulosic biofuel production will require an integrated strategy combining fungal-based biomass deconstruction, engineered fermentative partners, and optimized bioprocess control. Rather than relying on a single technological solution, future platforms will likely depend on the coordinated application of microbial ecology, metabolic engineering, and process engineering to achieve robust, scalable, and economically viable biofuel production.

6. Engineering Enzymes for More Effective Production

Filamentous fungi are considered excellent self-sustaining factories because of their versatile metabolism and efficient secretion systems, making them highly valuable in biotechnology. They are widely employed in the production of primary and secondary metabolites, large-scale enzyme and protein manufacturing, as well as in agriculture, bioenergy, and healthcare. However, a persistent challenge in developing filamentous fungal platforms for industrial purposes is the relatively low yield of heterologous proteins and metabolites [146]. An ideal model strain for biofuel production must be capable of utilizing large quantities of substrate, possess fast and deregulated sugar transport mechanisms, tolerate inhibitory compounds and final products, and maintain high metabolic fluxes to ensure greater ethanol productivity [145]. For a microbial genetic transformation tool to be suitable for industrial application, the expression system requires key features, including a host strain with a proven record of safe use, robust growth, and high protein productivity in low-cost media under industrial conditions [147].

Strain improvement is usually achieved through either classical or genetic engineering strategies. Classical techniques such as induced mutations, clonal selection, sexual or artificial hybridization, and evolutionary engineering can enhance strains without requiring detailed molecular knowledge of the organism, while genetic engineering approaches such as targeted gene deletions, gene regulation, pooled-segregant whole-genome analysis, and CRISPR/Cas9 systems allow precise modifications of microbial performance [148]. Both

methods offer valuable benefits but also present drawbacks, which random mutagenesis may introduce undesirable mutations leading to phenotypic instability, whereas recombinant DNA technology generates genetically modified organisms that face regulatory and environmental restrictions in some regions [149].

According to Table 2, a genetically engineered strain of *Myceliophthora thermophila* ATCC 42464 showed exceptional ethanol production capacity through metabolic engineering designed to mimic the Crabtree effect. Under consolidated bioprocessing conditions, this strain produced 52.8 g/L ethanol from cellulose and 39.8 g/L from corncob without external cellulase supplementation, whereas the parental wild-type strain generated almost no ethanol under the same conditions [150]. This highlights the effectiveness of targeted genetic modifications in improving ethanol biosynthesis and substrate conversion. In contrast, *Neurospora intermedia* CBS 131.92, a wild-type strain, yielded 32.2 g/L ethanol and 19.2 g/L biomass when cultivated on enzymatically liquefied bread waste at 150 g/L solids, with the fungal biomass containing approximately 45% protein [82]. Although its ethanol output is lower compared with the engineered *M. thermophila*, the performance of *N. intermedia* demonstrates the potential of unmodified filamentous fungi to valorize diverse substrates while simultaneously generating protein-rich biomass suitable for feed or food purposes.

Overall, these examples underscore the contrast between engineered and wild strains. Engineered fungi achieve superior ethanol titers by means of advanced metabolic redesign, whereas wild strains exhibit natural adaptability in processing varied waste materials, thereby supporting the development of integrated biorefinery systems.

Based on Table 2, engineered filamentous fungi cannot yet be considered to have reached industrial 2G ethanol yields. Metabolic engineering has improved microorganisms' ability to utilize pentose sugars such as xylose and arabinose, which are important components of lignocellulosic biomass, but the review by [103] shows that most successful engineering efforts have focused on yeasts and bacteria rather than filamentous fungi. Filamentous fungi remain promising because they can produce lignocellulolytic enzymes and potentially ferment both hexose and pentose sugars; however, their industrial application is limited by slow fermentation, inefficient pentose uptake, cofactor imbalance, inhibitor sensitivity, by-product formation, and difficulty in simultaneously fermenting C5 and C6 sugars. Consequently, although metabolic engineering provides useful strategies for improving fungal ethanol production, the available evidence does not yet demonstrate the high titer, productivity, stability, and mixed-sugar conversion required for industrial 2G ethanol production.

However, engineered *S. cerevisiae* with double-integrated XYL2 genes shows stronger potential for industrial 2G ethanol production than filamentous fungi that have only been screened for cellulase activity. The additional XYL2 copies improve xylose metabolism by enhancing the conversion of xylitol toward xylulose, helping the yeast consume xylose together with glucose and other hexose sugars from steam-exploded lignocellulosic biomass [151]. This co-consumption is important because conventional yeast preferentially consumes glucose and often leaves xylose unused, reducing the overall ethanol yield. Nevertheless, improved sugar utilization does not by itself prove that the strain has reached industrial performance; industrial evaluation also requires high ethanol titer, productivity, inhibitor tolerance, genetic stability, and consistent performance in realistic hydrolysates and large-scale fermenters. Therefore, the study demonstrates a promising metabolic-engineering strategy for approaching industrial 2G ethanol production, but the engineered strain should be described as industrially promising rather than conclusively industrial-ready unless the reported fermentation results meet those scale-up criteria.

Table 2. Comparison of ethanol yield from different filamentous fungi (native and engineered strains).

Fungi	Strain Type	Substrate	Ethanol Yield	Notes	References
Mucor indicus DSM 2185	Wild type	Corn cobs	16.46 g/L	Shows strong performance in integrated SSF with enzyme supplementation.	[152]
Rhizopus oryzae ATCC 9363	Wild type	Potato peel waste	18.83 g/L	Good for starchy waste; produces mixed products (ethanol + lactic acid).	[153]
Rhizopus arrhizus W4	Wild type	Glutinous rice	16.9 g/L	Screening can find high-producing isolates.	[154]
Fusarium oxysporum HG19	Wild type	Freshly grounded *Ficus* fruits	3.88 g/L	This strain displayed a single-step conversion of biomass that indicates its suitability as an ideal biocatalyst for the CBP approach from Ficus fruits.	[99]
Byssochlamys fulva AM130	Wild type	Rice straw	11.84 g/L	Utilize both glucose and xylose and produce ethanol.	[155]
Neurospora intermedia CBS 131.92	Wild type	Bread-waste medium	32.2 g/L	Edible filamentous fungi can be applied for efficient conversion of bread residues into value-added products such as ethanol.	[82]
Trametes hirsuta Bm-2	Wild type	Raw flour	13 g/L	Starch-rich ramon flour in a CBP study	[156]
Myceliophthora thermophila ATCC 42464	Engineered strain	Corn cob	39.8 g/L	Directly ferment cellulose into ethanol by mimicking the aerobic ethanol fermentation of yeast (the Crabtree effect)	[150]

6.1. Classical Metabolic Engineering of Fungi

Classical or rational metabolic engineering, which follows a top-down strategy, involves targeted genetic modifications in an organism's biochemical network. This requires detailed understanding of the genetic framework and metabolic pathways under study [157]. Such approaches usually rely on the overexpression or deletion of specific fermentative genes to enhance the rapid and efficient conversion of sugars into ethanol with higher recovery yields [158]. In filamentous fungi, one effective approach to increase protein expression and secretion is the optimization of transcriptional processes and codon usage of the desired protein [159]. Genetic modifications in *S. cerevisiae* have been directed towards introducing xylose utilization pathways, but these attempts have shown limited success. Current obstacles include glucose repression, inefficient xylose transport, redox imbalance in the xylose reductase/xylitol dehydrogenase system, poor expression of heterologous xylose isomerase, weak downstream pathways, and overall low ethanol output [20].

A good example is seen in *T. reesei* QM6a, from which the mutant QM9414 was derived. This strain displayed cellulase activity nearly four times higher than the original wild-type strain. From QM9414, another hypercellulolytic strain known as RUT-C30 was developed, and this mutant has become one of the most widely used strains in industrial processes involving biomass hydrolysis [160].

Classical Random Mutagenesis

A conventional approach for altering fungal genomes is the use of mutagenic agents such as ultraviolet irradiation, chemical compounds, including ethyl methanesulfonate (EMS) or nitroglycerin, as well as atmospheric and room temperature plasma (ARTP), all of which create random changes in DNA [161]. Although this technique lacks precision, random mutagenesis remains a widely applied method to improve microbial cellulase production to levels that are industrially viable. This practice continues to be a major strategy for enhancing cellulase yields in fungal strains [162].

The well-known *T. reesei* strain Rut-C30, a hyper-producer of cellulase, was created through three consecutive mutagenesis cycles starting from the wild-type QM6a, which was originally isolated in the Solomon Islands during World War II [163]. Rut-C30 carries a large chromosomal deletion of approximately 85 kb and has a truncated cre1 gene. The cre1 gene encodes a DNA-binding protein involved in carbon catabolite repression of cellulase genes in the presence of glucose. These genetic modifications allow Rut-C30 to reach extracellular protein levels up to 20 g/L [164]. In addition to its effective carbon transport systems and inducible cellulase genes, Rut-C30 also exhibits a strong protein secretion system. Studies revealed that Rut-C30 secreted about 2.7 times more protein than QM6a when cultivated on 6% roll-milled cotton [165,166].

6.2. Genetic Modification and Metabolic Engineering of Fungi

Genetic modification and metabolic engineering have become indispensable strategies for developing filamentous fungi as efficient platforms for second-generation (2G) bioethanol production. Unlike conventional strain improvement approaches based on random mutagenesis, modern genetic engineering enables targeted manipulation of genes associated with lignocellulose degradation, sugar transport, central carbon metabolism, redox regulation, and stress tolerance. These approaches aim not only to enhance extracellular enzyme production but also to improve intracellular carbon utilization by overcoming metabolic constraints that limit lignocellulosic fermentation efficiency [167].

Current engineering strategies for 2G bioethanol production generally involve two complementary approaches: (i) improving the hydrolytic capacity of cellulolytic fungi through enhanced production of cellulases, hemicellulases, and accessory enzymes, and

(ii) engineering ethanologenic microorganisms to improve the conversion of both hexose and pentose sugars into ethanol [35,168]. However, increased lignocellulolytic enzyme production alone does not necessarily translate into higher ethanol productivity because intracellular limitations, including inefficient sugar transport, carbon catabolite repression, cofactor imbalance, precursor competition, and by-product formation, can restrict the conversion of released sugars into ethanol.

A major goal of fungal metabolic engineering is to redirect carbon flux from lignocellulose-derived sugars toward ethanol biosynthesis. Following biomass hydrolysis, glucose, xylose, and arabinose must be transported into fungal cells before entering glycolysis and the pentose phosphate pathway, ultimately generating pyruvate for ethanol production. However, inefficient sugar uptake, preferential glucose utilization, and competition between C5 and C6 sugar transport systems represent major barriers to efficient co-fermentation. Therefore, engineering sugar transport pathways through promoter modification, transporter engineering, or heterologous expression has emerged as a promising strategy to improve intracellular sugar availability and enhance carbon flow toward ethanol formation [169]. In addition, reducing carbon diversion into competing pathways, including organic acid production, glycerol formation, and excessive biomass synthesis, may increase pyruvate availability for ethanol biosynthesis.

Redox imbalance represents another major metabolic challenge, particularly during pentose fermentation. In the conventional xylose reductase/xylitol dehydrogenase (XR/XDH) pathway, xylose reductase generally utilizes NADPH to convert xylose into xylitol, whereas xylitol dehydrogenase requires NAD^+ for conversion of xylitol into xylulose. This cofactor imbalance can result in inefficient regeneration of reducing equivalents, xylitol accumulation, and reduced ethanol productivity. Moreover, lignocellulosic hydrolysates contain inhibitory compounds such as furfural, 5-hydroxymethylfurfural (HMF), weak organic acids, and phenolic compounds generated during pretreatment. Detoxification of these inhibitors requires additional NAD(P)H-dependent reactions, thereby competing with xylose metabolism and further disrupting intracellular redox balance [170]. Consequently, metabolic engineering strategies increasingly focus on improving cofactor regeneration, modifying XR/XDH cofactor preference, enhancing antioxidant capacity, and increasing tolerance toward fermentation inhibitors.

Quantitative evaluation of metabolic engineering strategies requires integration with systems biology approaches, including ^{13}C-metabolic flux analysis (^{13}C-MFA), flux balance analysis (FBA), genome-scale metabolic models (GEMs), and kinetic modeling. Unlike transcriptomic and proteomic analyses, which primarily describe changes in gene expression or protein abundance, metabolic flux approaches provide insight into how genetic modifications influence intracellular carbon distribution. ^{13}C-MFA integrates isotope-labeling patterns with extracellular substrate uptake and product secretion rates to estimate carbon flow through glycolysis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, biomass formation, and ethanol synthesis. Meanwhile, FBA and GEMs provide predictive frameworks to identify metabolic control points and prioritize candidate reactions for overexpression, repression, or deletion. Kinetic models further complement these approaches by describing dynamic relationships among enzyme capacity, substrate availability, cofactor regeneration, product inhibition, and cultivation conditions. Together, these approaches allow researchers to determine whether genetic modifications increase productive ethanol flux rather than merely altering metabolite concentrations.

The importance of balancing carbon flux and redox metabolism has been demonstrated in *M. thermophila*. Modification of NADH metabolism through disruption of NADH-consuming pathways, including glycerol-3-phosphate dehydrogenase and mitochondrial external NADH dehydrogenases, increased reducing-equivalent availability for L-malic

acid production [171]. However, excessive NADH accumulation caused oxidative stress and impaired fungal growth, which was alleviated through overexpression of the transhydrogenase SthA and optimization of UTR1 expression. These findings demonstrate that increasing reducing-equivalent availability must be balanced with cofactor regeneration, oxidative stress control, and cellular growth requirements. Furthermore, ^{13}C-MFA analysis of engineered *M. thermophila* strains revealed altered carbon redistribution associated with enhanced malic acid production, including increased glucose uptake, enhanced glycolytic and TCA-cycle fluxes, and reduced oxidative phosphorylation activity [172]. Although these studies focused on organic acid production rather than ethanol synthesis, they provide an important methodological framework for evaluating intracellular flux redistribution in engineered filamentous fungi. Similar approaches could be applied to determine whether modifications targeting sugar transport, pyruvate metabolism, alcohol dehydrogenase activity, or competing pathways enhance ethanol-producing flux.

A representative example of pathway engineering for fungal ethanol production was demonstrated in *M. thermophila*. Introduction of the heterologous *S. cerevisiae* ADH1 gene increased glucose-derived ethanol production by approximately 57%, indicating that alcohol dehydrogenase activity contributed to ethanol-forming limitations. However, this modification did not improve cellobiose-derived ethanol production, suggesting that substrate uptake and intracellular processing represented stronger bottlenecks during cellobiose utilization. In contrast, expression of the *N. crassa* glucose transporter GLT-1 increased glucose-derived ethanol production by 131%, whereas introduction of cellodextrin transporters CDT-1/CDT-2 improved cellobiose-derived ethanol production by approximately 200%. Further reduction of *pyc* expression increased ethanol production by an additional 23%, reaching 11.3 g L^{-1}, likely by reducing pyruvate diversion toward oxaloacetate formation and increasing pyruvate availability for ethanol synthesis [81]. Nevertheless, direct intracellular flux measurements are still required to confirm whether these modifications enhance glycolytic flux, pyruvate-to-ethanol conversion, or alter competing metabolic pathways.

Despite these advances, most genetically engineered filamentous fungi remain evaluated primarily at laboratory scale, and quantitative relationships between genetic modifications and intracellular metabolic redistribution remain insufficiently characterized. Future research should integrate genome editing with ^{13}C-MFA, dynamic FBA, kinetic modeling, multi-omics analysis, and adaptive laboratory evolution to identify true metabolic control points governing ethanol productivity. Such integrated systems-metabolic engineering approaches will enable the rational development of robust fungal cell factories with improved sugar utilization, inhibitor tolerance, and bioethanol production efficiency from diverse lignocellulosic feedstocks.

6.2.1. Transcription Factors as Regulatory Proteins

In fungi, the expression of cellulase genes is primarily controlled at the transcriptional level through pathway-specific transcription factors, most of which are members of the Zn(II)2Cys6 family of zinc binuclear cluster proteins [173]. Both activators, such as Xyr1, XlnR, ACE II, LAE1, and BglR, and repressors, including Cre1, CreA, ACE1, and RCEI, coordinate the regulation of lignocellulolytic enzyme gene expression [99]. Transcription factors play a crucial role in governing the activity of genes associated with biomass degradation. These regulatory proteins bind to the promoter regions of target genes, including CAZymes, sugar transporters, signaling proteins, and other transcription factors, in order to either activate or suppress their transcription, with their activity influenced by nutritional and environmental signals such as the availability of carbon, pH level, and nitrogen sources [174].

Genetic engineering has become a widely applied strategy in eukaryotes to manipulate transcription factors. In recent years, considerable progress has been made in modifying endogenous transcription factors to reprogram transcriptional regulatory networks and enhance cellulase production in *T. reesei* [175]. Altering transcription factors is recognized as an effective approach to boost enzyme production in fungi. For instance, Xyr1 acts as a positive regulator of cellulase induction, and its deletion eliminates induction responses entirely, whereas its overexpression significantly enhances cellulase synthesis [176].

6.2.2. Synthetic Transcription Factors (STFs)

Synthetic transcription factors (STFs) provide a programmable approach for rewiring fungal transcriptional networks by combining a DNA-binding domain (DBD) derived from a native transcription factor with an activation or repression domain [145]. Through recognition of specific upstream activating sequences (UAS) within synthetic or native promoters, STFs enable controlled modulation of transcriptional strength by altering promoter architecture, transcription-factor abundance, or inducible regulatory systems. Compared with conventional gene overexpression strategies, STFs provide greater flexibility by allowing dynamic regulation of target genes according to environmental signals or metabolic requirements [177].

Several STF-based strategies have been developed to enhance lignocellulolytic enzyme production in filamentous fungi. For example, fusion of the DNA-binding domain of the carbon catabolite repressor CreA/Cre1 with the cellulase regulator Xyr1 generated a synthetic regulator that enhanced cellulase production in a CreA/Cre1-deficient *T. reesei* strain cultivated on glucose [178,179]. Similarly, targeted modification of Xyr1 regulatory regions enabled cellulase and hemicellulase expression under non-inducing carbon conditions, demonstrating that transcriptional rewiring can partially overcome carbon catabolite repression and improve enzyme production [180]. In another study, quantitative tuning of Xyr1 expression increased cellulase activity by 3.2-fold and improved saccharification efficiency of acid-pretreated corncob residues by 57.2% in the optimized strain [181]. These findings demonstrate that precise regulation of transcription-factor dosage can significantly influence extracellular enzyme production. However, increased expression of cellulolytic genes does not necessarily translate directly into higher ethanol productivity, as STF-mediated regulatory changes may also affect downstream processes, including sugar transport, carbon allocation, cofactor balance, respiration, and secondary metabolism.

Therefore, the quantitative impact of STFs on fungal metabolic networks must be evaluated beyond individual target-gene expression or enzyme production levels. A systems-level analysis in *Aspergillus nidulans* demonstrated that fungal transcriptional regulators coordinate interconnected metabolic pathways, including glycolysis, the pentose phosphate pathway, ethanol metabolism, gluconeogenesis, and NADPH-generating reactions in response to different carbon sources [182]. These findings highlight that transcription factors operate within hierarchical regulatory networks, where manipulation of a single regulatory node may generate both beneficial and unintended effects on cellular metabolism. Consequently, STF-mediated activation of cellulolytic genes, sugar transporters, or ethanol-associated pathways should be assessed using integrated measurements, including transcriptomics, proteomics, enzyme activity assays, sugar uptake rates, ethanol and by-product formation, and intracellular NAD(P)H/NAD(P)$^+$ ratios. Such analyses are necessary to determine whether STF-driven regulatory changes increase productive carbon flux toward ethanol formation or merely enhance intermediate phenotypes such as enzyme secretion.

Recent advances in fungal regulatory biology provide opportunities for developing next-generation STFs targeting multiple metabolic layers. Positive regulators, including Xyr1/XlnR, Ace2, and Ace3, activate genes encoding lignocellulose-degrading enzymes, whereas repressors such as Cre1/CreA and Ace1 regulate carbon catabolite repression and pathway limitation [66]. Engineering synthetic regulators that coordinate these regulatory networks may enable simultaneous enhancement of enzyme production, sugar utilization, and ethanol biosynthetic capacity. However, successful application of STFs for industrial 2G bioethanol production requires integration with metabolic engineering and quantitative modeling approaches. For example, STF-mediated activation of cellulase regulators may increase sugar release from lignocellulosic biomass, but ethanol productivity will ultimately depend on downstream metabolic capacities, including sugar transport, glycolytic flux, pyruvate conversion, and redox balance. Therefore, coupling STF-based regulatory engineering with ^{13}C-metabolic flux analysis (^{13}C-MFA), flux balance analysis (FBA), and kinetic modelling will be essential to establish quantitative relationships between transcriptional rewiring and intracellular carbon redistribution, enabling the rational design of predictive fungal cell factories.

6.2.3. Technology of CRISPR/Cas9 System

Limited genetic tractability remains one of the major barriers to developing industrially competitive filamentous fungi for second-generation (2G) bioethanol production. Conventional genome engineering in filamentous fungi is often slow, labor-intensive, and constrained by the limited availability of selectable markers, making iterative strain improvement difficult. Among the available genome-editing technologies, the CRISPR/Cas9 system has emerged as the most versatile and widely adopted tool for fungal genetic engineering due to its high precision, multiplex editing capability, and ease of implementation. The abundance of protospacer adjacent motif (PAM) sequences in fungal genomes further facilitates its application across diverse filamentous fungal species [66,183].

The ability of CRISPR/Cas9 to introduce multiple genetic modifications simultaneously has accelerated rational strain engineering for enhanced bioethanol production. For example, multiplex genome editing in the thermophilic fungus *Myceliophthora* enabled the simultaneous modification of four target genes, resulting in a 13-fold increase in cellulase production [184]. Such advances demonstrate the potential of CRISPR/Cas9 to improve key industrial traits, including cellulase secretion, carbon utilization, stress tolerance, and metabolic flux distribution. Compared with conventional transformation approaches, CRISPR/Cas9 enables precise gene disruption, targeted gene replacement, pathway engineering, and multiplex genome editing, while strategies such as Cas9–sgRNA ribonucleoprotein delivery, enhanced homologous recombination, and suppression of non-homologous end joining (NHEJ) further improve editing efficiency [184].

Despite these advantages, CRISPR/Cas9 should be regarded as an enabling technology rather than a complete solution for filamentous fungal engineering. Biological characteristics intrinsic to many filamentous fungi, including multinucleate hyphae, heterokaryosis, persistence of unedited nuclei, and relatively low homologous recombination efficiency, continue to complicate the generation of genetically homogeneous strains. In addition, the efficiency of CRISPR/Cas9 remains highly strain-dependent, and several technical limitations persist, including low transformation and regeneration efficiencies, PAM-sequence constraints, residual off-target effects, inefficient DNA repair, and challenges associated with obtaining stable transformants [185]. These factors continue to restrict the reproducibility and scalability of genome engineering in industrial fungal strains.

From an industrial perspective, CRISPR/Cas9 substantially strengthens the capacity to address key bottlenecks associated with filamentous fungi, including improving cellulase production, optimizing sugar-utilization pathways, minimizing by-product formation, and enhancing tolerance to fermentation inhibitors and environmental stresses [186]. However, most CRISPR-engineered strains have been evaluated under laboratory-scale conditions, with relatively few studies demonstrating consistent performance under pilot-scale or industrial fermentation environments. Consequently, improvements in genome-editing efficiency do not necessarily translate into commercially competitive ethanol titers, productivities, pentose co-fermentation capabilities, long-term genetic stability, or robust large-scale bioprocess performance.

Collectively, current evidence indicates that CRISPR/Cas9 has transformed the genetic engineering of filamentous fungi by enabling rapid and precise strain development. Nevertheless, its successful application to industrial 2G bioethanol production will depend on its integration with systems biology, metabolic engineering, adaptive laboratory evolution, and process optimization. Therefore, CRISPR/Cas9 should be viewed as a foundational platform that facilitates strain improvement rather than a standalone technology capable of overcoming the multifaceted biological, engineering, and economic challenges associated with commercial lignocellulosic bioethanol production.

Modern engineering approaches, including CRISPR/Cas9 genome editing, synthetic transcription factors (STFs), adaptive laboratory evolution (ALE), genome-scale metabolic engineering, and AI-assisted systems biology, target multiple cellular processes involved in lignocellulose conversion and ethanol biosynthesis. Key molecular targets include cellulase expression, pentose utilization pathways, redox homeostasis, stress-response signaling, carbon flux distribution, and fungal morphology. Collectively, these complementary strategies enable the rational optimization of fungal metabolism to improve lignocellulose degradation, inhibitor tolerance, ethanol productivity, and process robustness. The integration of these engineering tools, molecular targets, key metabolic pathways, and their associated outcomes is summarized schematically in Figure 3.

6.3. Intracellular Metabolic Bottlenecks Limiting Carbon Flux Toward Ethanol Production

The gap between theoretical and actual ethanol yields in filamentous fungi is determined not only by substrate utilization but also by intracellular metabolic constraints, particularly redox imbalance and carbon flux partitioning. During pentose metabolism, the xylose reductase–xylitol dehydrogenase (XR–XDH) pathway creates an intrinsic cofactor imbalance because xylose reductase preferentially utilizes NADPH, whereas xylitol dehydrogenase generates NADH. This mismatch promotes xylitol accumulation, reduces ethanol productivity, and diverts carbon away from ethanol formation, especially under oxygen-limited conditions. Furthermore, the oxidative pentose phosphate pathway (PPP), a major source of NADPH for biosynthesis and detoxification, competes with glycolysis for carbon while releasing CO_2, thereby lowering the overall carbon conversion efficiency. Recent metabolic engineering studies in *S. cerevisiae* demonstrated that reconstructing alternative NADPH regeneration pathways significantly improved xylose utilization and ethanol production by alleviating redox imbalance [187]. Although demonstrated in yeast, these findings illustrate a general metabolic principle that is equally relevant to filamentous fungi.

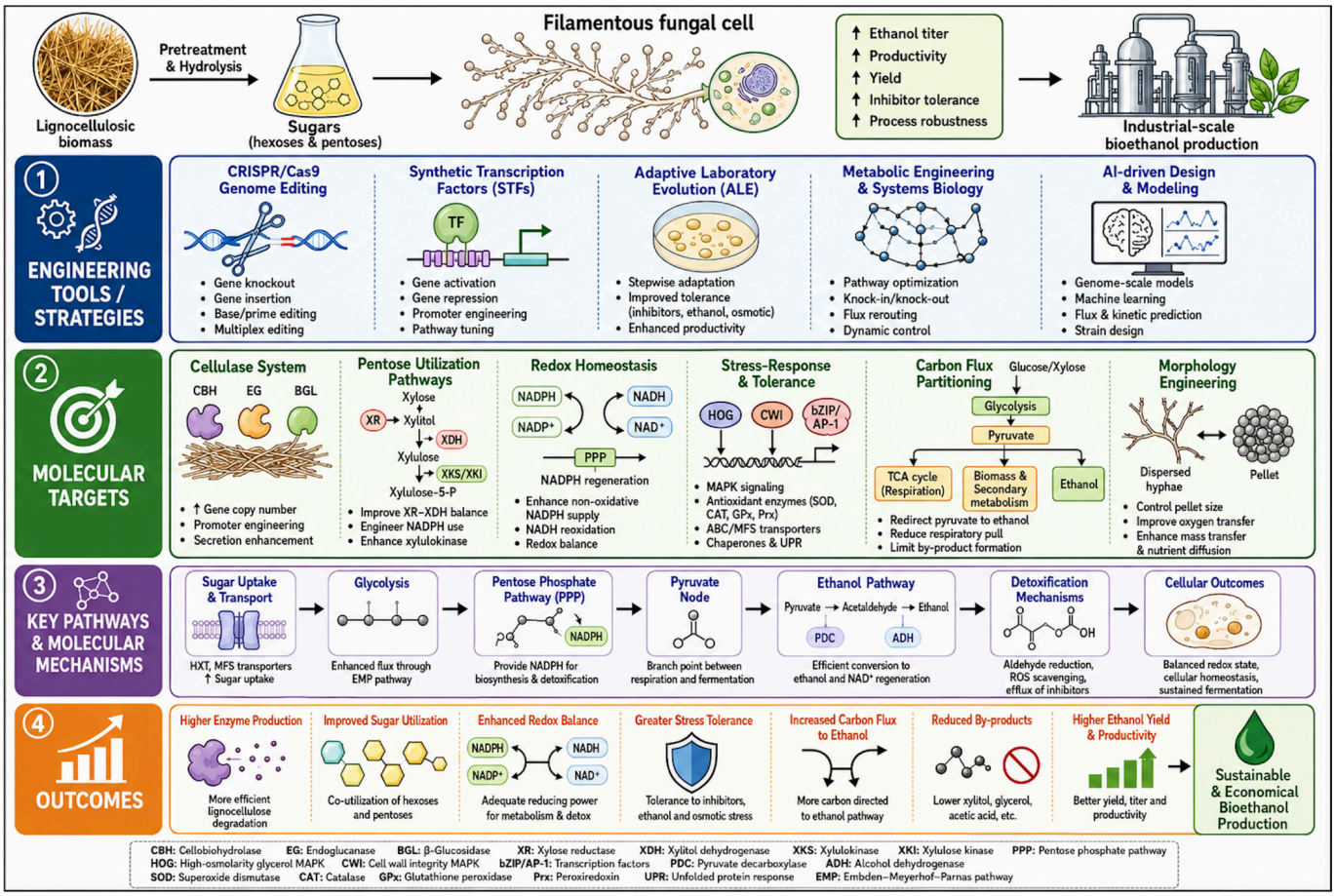


Figure 3. Overview of genetic and metabolic engineering strategies for improving bioethanol production in filamentous fungi.

In *F. oxysporum*, ethanol production is further constrained because NADPH is simultaneously required for inhibitor detoxification and cellular biosynthesis, whereas NADH must be continuously regenerated to sustain glycolysis and alcohol dehydrogenase activity. Under lignocellulosic hydrolysate stress, increased demand for reducing equivalents shifts cellular resources from ethanol production toward survival and maintenance. In contrast, *T. reesei* naturally directs a large proportion of carbon toward respiratory metabolism, biomass formation, cellulase production, and secondary metabolism rather than fermentation. Consequently, pyruvate serves as a key metabolic branch point where competition between respiration and fermentative pathways limits ethanol production [188]. Collectively, these intracellular bottlenecks indicate that improving ethanol yield will require coordinated engineering of redox homeostasis, carbon flux distribution, and metabolic pathway regulation rather than simply enhancing glycolytic activity or overexpressing fermentative enzymes.

7. Challenges in Biofuel Production

Despite their enzymatic versatility, several challenges hinder the large-scale application of filamentous fungi in bioethanol production. One of the foremost obstacles is lignocellulose recalcitrance and dependence on pretreatment. Agricultural residues, forestry by-products, and food waste differ considerably in cellulose content, lignin structure, and inhibitory compound profiles, which complicates fungal utilization. The diversity and complexity of lignocellulosic substrates often require tailored fungal strains and optimization of culture conditions, leading to increased research and development costs. Moreover, pretreatment and hydrolysis steps frequently release inhibitory compounds

such as furfural, HMF, and acetic acid, which can impair fungal growth, reduce enzyme activity, and lower ethanol yields [186].

Another major limitation is the low native ethanologenic capacity of filamentous fungi, which often rely on respiratory rather than fermentative metabolism. Unlike *S. cerevisiae*, which naturally channels sugars into ethanol, most filamentous fungi preferentially direct carbon flux toward biomass and secondary metabolites. This inherent metabolic bias results in low ethanol titers unless the strains are genetically engineered to mimic yeast-like fermentation pathways. In addition, the growth rate of filamentous fungi is slower compared to bacterial and yeast systems such as *Escherichia coli* or *S. cerevisiae*, which further limits their competitiveness in industrial-scale biofuel production. Growth morphology also plays a role, as filamentous networks can cause variable mass transfer and product yields in submerged fermentations [183].

A further challenge is the low ethanol tolerance of filamentous fungi, as many strains are unable to grow in ethanol concentrations exceeding 30 to 40 g/L. For instance, [81] reported that mycelia grew moderately in medium containing 30 g/L ethanol but failed to grow at 40 g/L. This sensitivity restricts their ability to achieve high product titers during fermentation and makes them less robust under industrial conditions. Filamentous fungi pose unique challenges compared to unicellular systems such as yeast and bacteria. These challenges include technical difficulties in transformation, diverse morphologies and life states, limited selectable markers, and incomplete genomic resources [183]. Although strategies such as adaptive laboratory evolution and metabolic engineering have been applied to improve tolerance, their use remains largely restricted to a few model strains. While *T. reesei* and *A. niger* benefit from more mature CRISPR-based systems, many industrially relevant fungi remain recalcitrant to genetic manipulation, and wild strains often produce high levels of secondary metabolites that complicate product isolation. A persistent barrier is the low frequency of homologous recombination at target loci, as demonstrated in *Penicillium digitatum*, where CRISPR/Cas9 has improved gene editing but still shows relatively low efficiencies requiring further optimization [189]. Collectively, these limitations highlight that, compared with well-established bacterial and yeast platforms, filamentous fungi still lag behind in engineering efficiency, standardization, and scalability. As summarized in Table 3, the key challenges include substrate recalcitrance and pretreatment dependence, low native ethanologenic capacity and slower growth rate, low ethanol tolerance, and limited availability of genetic engineering tools. Collectively, these barriers highlight the need for integrated strategies that combine strain improvement, process optimization, and systems biology approaches. Addressing these limitations will be essential for realizing the full potential of filamentous fungi in next-generation bioethanol production and broader biorefinery applications.

Table 3. Challenges and solutions to produce bioethanol using filamentous fungi.

Challenges	Main Problem	Proposed Solutions	References
Lignocellulose recalcitrance and pretreatment dependence	Raw biomass crystalline cellulose and lignin limit enzyme accessibility; pretreated feedstocks add cost and inhibitors that impair fermentation.	Improve secreted ligninolytic/cellulolytic cocktails; engineer tolerance to inhibitors (furfural, HMF, phenolics); integrate mild pretreatment (e.g., biological, ionic liquids) and simultaneous saccharification/fermentation (SSF/CBP) strategies.	[190]
Low native ethanologenic capacity/respiratory metabolism	The main challenge in ethanol fermentation by cellulosic fungi lies in their difficulty to perform under anaerobic conditions, as they are naturally aerobic microorganisms.	If a Crabtree-like effect could be engineered in these fungi through molecular modifications, it may enable efficient ethanol production directly from cellulose.	[150]
Low ethanol tolerance	Ethanol is toxic at moderate concentrations	Enhancing the ethanol tolerance of these fungi and potential chassis cells can be achieved through multiple strategies, including adaptive laboratory evolution (ALE), genetic modifications, and membrane engineering approaches such as altering fatty acid chain length and adjusting the ratio of unsaturated fatty acids.	[96]
Limited genetic tools/transformation efficiencies for some species	Compared with *S. cerevisiae*, many filamentous fungi lacked robust CRISPR/marker systems until recently, which slowed progress in complex engineering. The first reports of CRISPR/Cas9-mediated genome editing in filamentous fungi were published in 2015.	To accelerate strain development, genome engineering approaches such as CRISPR/Cas9 can be leveraged to introduce targeted deletions, insertions, or point mutations, while future progress will require the adoption of CRISPR/Cas9 multiplexing, stronger promoters, marker recycling systems, and expanded genomic toolkits tailored for industrial filamentous fungi.	[147]

Future Prospects of Filamentous Fungi in Bioethanol Production

Ubiquitous in nature as decomposers with complex enzyme systems, filamentous fungi possess unique physiological and metabolic traits that enable them to convert agricultural residues and food processing by-products into high-value bio-based products [191]. Despite their ecological and industrial importance, these fungi have historically been underutilized in synthetic biology due to their multicellular complexity, slow growth rates, and limited molecular toolkits [83]. The convergence of omics technologies, artificial intelligence–driven metabolic modeling, and high-throughput screening, however, is rapidly transforming fungal biotechnology into a powerful platform for sustainable bioethanol production.

Future advancements are expected to focus on discovering novel fungal species with superior lignocellulolytic activity and engineering hybrid metabolic pathways capable of simultaneous saccharification and fermentation. Integrating genome-editing tools such as CRISPR/Cas9 with systems biology will enable the design of "smart" fungal strains optimized for enhanced ethanol yields, inhibitor tolerance, and efficient substrate utilization [162]. Co-cultivation strategies that combine filamentous fungi with ethanologenic yeasts may further enhance process efficiency by coupling enzymatic hydrolysis with rapid fermentation in a single bioprocess.

However, achieving predictable and scalable performance from these engineered fungal systems remains challenging due to the complexity of fungal metabolism, regulatory networks, and bioprocess conditions. Emerging artificial intelligence (AI)-driven computational approaches, particularly machine learning (ML) and operator-learning frameworks, provide new opportunities to overcome these limitations by enabling predictive rather than empirical optimization strategies. These methods enable the development of rapid and generalizable surrogate models for nonlinear differential-equation systems, including those describing dynamic cellular behavior and metabolic regulation. In parallel, large language models (LLMs) are increasingly being explored for multi-scale biological reasoning. For example, MS-CoTF (Multi-scale Chain-of-Thought Fusion) [192] integrates molecular, cellular, and system-level information to improve interpretability and reasoning in complex biological tasks. Such developments align with emerging hybrid modeling frameworks that combine mechanistic knowledge, such as physics-informed or universal differential equations, with neural networks capable of capturing previously unresolved regulatory interactions. For filamentous fungi involved in 2G ethanol production, these approaches provide a potential framework for integrating transcriptomic, metabolomic, and kinetic datasets to predict stress-response dynamics under diverse fermentation conditions, including variations in furan and phenolic inhibitor concentrations, ethanol accumulation, and carbon source availability. Ultimately, such predictive models could support environment-aware strain engineering by identifying robust genetic targets without requiring complete model redevelopment for every hydrolysate composition. For filamentous fungi, this capability is particularly valuable because inhibitor tolerance, enzyme secretion, morphology, and ethanol formation are governed by interconnected regulatory networks that are difficult to optimize through conventional experimental approaches alone.

Machine learning is also increasingly being integrated with genome-scale metabolic models (GEMs) to improve metabolic reconstruction, refine reaction networks, and generate condition-specific predictions from multi-omics datasets. ML and deep learning approaches can enhance multiple stages of GEM development, including gap filling, annotation refinement, and integration of transcriptomic and metabolomic information to improve prediction of metabolic phenotypes under different environmental conditions [193]. In filamentous fungi used for 2G ethanol production, this ML-enhanced GEM framework could enable the construction of inhibitor-aware metabolic models by integrating omics

profiles from hydrolysate-stressed cultures of industrially relevant fungi such as *Aspergillus* and *Trichoderma*. These models could identify condition-dependent flux distributions, reveal metabolic bottlenecks associated with NADPH-demanding detoxification pathways, and predict engineering strategies that balance ethanol production, biomass formation, and stress tolerance. Specifically, ML approaches may assist in (i) estimating kinetic and enzymatic capacity constraints, such as enzyme turnover rates and transporter activities, to improve flux predictions under inhibitor stress; (ii) identifying active metabolic subnetworks from multi-omics datasets to construct context-specific GEMs; and (iii) linking model-derived features with predictive algorithms to forecast tolerance phenotypes and prioritize strain designs before experimental validation.

Although constraint-based GEMs provide valuable insights into metabolic potential, they are limited in their ability to capture rapid and transient cellular responses following exposure to lignocellulose-derived inhibitors. Recent developments in machine-learning-assisted parameter estimation, expanded kinetic databases, and generative modeling frameworks such as RENAISSANCE have facilitated the construction of large-scale kinetic models capable of capturing dynamic metabolic states and regulatory interactions [194]. For filamentous fungi, these advances offer the possibility of modelling time-dependent responses to hydrolysate stress, including changes in NADPH demand, redox cofactor balancing, aldehyde detoxification, and MAPK-mediated regulatory responses. By incorporating experimentally derived kinetic parameters, including enzyme catalytic rates, substrate affinity constants, and inhibitor coefficients, into enzyme-constrained or kinetic GEMs, researchers can simulate how furfural, HMF, weak acids, and phenolic compounds reshape metabolic flux distributions over fermentation time. Such predictive frameworks may enable in silico evaluation of environment-specific engineering strategies, including transporter enhancement, aldehyde reductase overexpression, or redox pathway optimization, while supporting rapid screening of strain–process combinations relevant to industrial-scale fungal bioreactors.

At the industrial process level, successful translation of engineered filamentous fungi from laboratory studies to commercial bioethanol production will require improved strategies for bioreactor scale-up and process control. Unlike unicellular microorganisms, filamentous fungi exhibit complex morphological transitions, including dispersed mycelial growth and pellet formation, which strongly influence broth rheology, oxygen transfer, nutrient diffusion, and enzyme secretion. These characteristics often result in significant differences between laboratory-scale performance and industrial fermentation outcomes. To overcome these challenges, AI-assisted digital twins integrating metabolic models, computational fluid dynamics (CFD), online sensor data, and kinetic simulations could provide predictive insights into scale-dependent behaviors such as oxygen limitation, substrate gradients, heat transfer, fungal morphology evolution, and ethanol productivity. Such predictive platforms would enable adaptive control of key operational parameters, including agitation, aeration, feeding strategies, and cultivation conditions, thereby reducing reliance on empirical optimization. Ultimately, integrating AI-driven modeling with advanced bioreactor monitoring could improve process robustness, reproducibility, and economic feasibility, accelerating the industrial deployment of filamentous fungi as sustainable bioethanol-producing platforms.

The role of bioethanol should also be assessed within the broader context of evolving renewable energy systems. Although electricity generation from solar, wind, and hydropower has made major strides, sectors such as aviation, maritime transport, and heavy logistics continue to face challenges in decarbonization due to their high energy density demands and long operational lifetimes [195]. While improvements in fuel efficiency and aircraft engine design can contribute to modest annual reductions in greenhouse

gas emissions, significant decarbonization will ultimately require large-scale adoption of biofuels. The incorporation of biofuels into the aviation sector could substantially reduce carbon dioxide emissions by 2050 [196]. Using bioethanol as a component of hybrid energy systems or as a feedstock for Sustainable Aviation Fuel (SAF) production can help preserve its strategic importance in achieving carbon-neutral transportation.

To address the SAF Grand Challenge, diverse bio-feedstocks must be supported by equally diverse conversion technologies. Currently, seven SAF production pathways have been approved under ASTM D7566, including gasification coupled with Fischer–Tropsch synthesis, hydroprocessed esters and fatty acids (HEFA), synthesized iso-paraffins (SIP), Fischer–Tropsch synthetic paraffinic kerosene with aromatics (FT-SPK/A), alcohol-to-jet (ATJ), catalytic hydrothermolysis (CH), and hydroprocessed hydrocarbons derived from esters and fatty acids (HH-SPK or HC-HEFA) [197]. Among these, HEFA fuels are the most established and widely implemented in aviation. They are produced by hydroprocessing lipid-rich feedstocks such as vegetable oils, animal fats, or microbial oils, during which oxygen and other heteroatoms are removed to generate hydrocarbons structurally similar to those in conventional jet fuels [198].

Oleaginous microorganisms serve as a renewable and sustainable source of lipid feedstocks for HEFA-based sustainable aviation fuel (SAF) production. They are capable of accumulating lipids that constitute 20–80% of their dry biomass, primarily in the form of triglycerides (TAGs) and free fatty acids, collectively known as single-cell oils (SCOs) [199]. Among these, filamentous fungi are particularly valued as feedstocks for biodiesel production due to the high degree of unsaturation in their lipids. Nevertheless, the large-scale industrial application of microbial SCOs remains challenging because of the high cost associated with their production [200].

The continued advancement of fungal bioethanol production will rely on the integration of metabolic engineering, automation, and cross-disciplinary renewable energy technologies. Aligning fungal-based bioprocesses with broader sustainable energy frameworks will ensure that bioethanol remains both relevant and competitive within next-generation renewable fuel systems. Collectively, these scientific and technological developments will define the next frontier of fungal-based biofuel innovation, reinforcing the role of filamentous fungi as key enablers in the global transition toward sustainable energy and a circular bioeconomy.

8. Conclusions

Filamentous fungi have emerged as versatile microbial platforms with considerable potential to advance sustainable second-generation (2G) bioethanol production and integrated biorefinery development. Their exceptional capacity to secrete diverse lignocellulolytic enzymes, degrade complex lignocellulosic biomass, and produce a wide range of value-added bioproducts distinguishes them from conventional ethanologenic microorganisms. Recent advances involving *T. reesei*, *A. niger*, *F.oxysporum*, and *N. crassa* have demonstrated significant progress in enzyme production, biomass saccharification, consolidated bioprocessing, and direct ethanol fermentation. Likewise, fungal–yeast co-culture systems have shown considerable promise for enhancing enzyme complementarity, substrate utilization, and ethanol productivity.

However, current evidence indicates that no single technological advancement is sufficient to achieve economically competitive 2G bioethanol production. Instead, the industrial competitiveness of filamentous fungi depends on overcoming several interconnected bottlenecks, including efficient lignocellulose deconstruction, tolerance to pretreatment-derived inhibitors, balanced enzyme production, high ethanol productivity, genetic tractability, and scalable bioprocesses. While advances in pretreatment technologies, enzyme engineer-

ing, microbial co-cultivation, metabolic engineering, and CRISPR/Cas9-mediated genome editing have substantially improved fungal performance, these strategies remain largely complementary rather than independently transformative. Their successful industrial implementation will require coordinated integration rather than isolated optimization.

Beyond bioethanol production, filamentous fungi are expected to play an increasingly important role in integrated biorefineries by enabling the simultaneous production of industrial enzymes, organic acids, microbial proteins, lipids, and lignin-derived biochemicals, thereby improving feedstock valorization, process economics, and environmental sustainability. Nevertheless, several challenges remain unresolved, including reducing enzyme production costs, improving inhibitor tolerance, achieving efficient pentose utilization, maintaining stable co-culture systems, and ensuring long-term strain stability under industrial fermentation conditions. Furthermore, most reported technologies have been validated only at laboratory scale, highlighting the need for pilot-scale demonstration and commercial verification.

Future research should therefore prioritize the integration of systems biology, multi-omics analyses, artificial intelligence-assisted strain engineering, adaptive laboratory evolution, and advanced bioprocess engineering to accelerate the development of robust fungal cell factories. Equally important, techno-economic analysis (TEA), life-cycle assessment (LCA), and pilot-scale validation should become integral components of future studies to evaluate commercial feasibility alongside technical performance. Such multidisciplinary approaches will help bridge the gap between laboratory innovation and industrial application.

Overall, filamentous fungi have evolved from being recognized primarily as producers of lignocellulolytic enzymes to becoming multifunctional microbial platforms capable of supporting sustainable biorefineries and circular bioeconomy systems. Their future success will not depend on any single breakthrough, but on the effective integration of biological innovation, genome engineering, enzyme technology, and scalable bioprocess design. As these complementary advances continue to converge, filamentous fungi are well positioned to become key contributors to economically viable second-generation bioethanol production and the broader transition toward a low-carbon, bio-based economy.

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Abbreviations

The following abbreviations are used in this manuscript:

CBP Consolidated bioprocessing
SSC Solid-state cultivation
SSF Solid-state fermentation
STF Synthetic transcription factors

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