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Fungal strain improvement for efficient cellulase production and lignocellulosic biorefinery: Current status and future prospects

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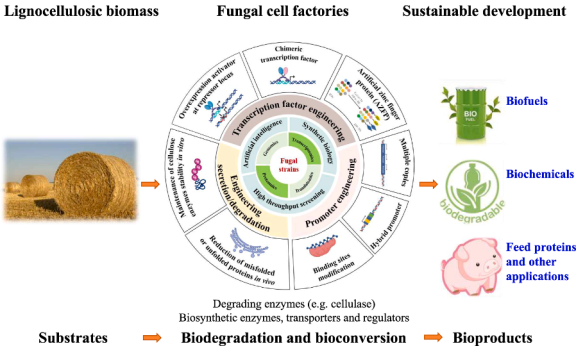
HIGHLIGHTS

- Crude enzymes produced by fungal cell factories benefit economic biomass degradation.
- Filamentous fungi are common microbial cell factories for producing cellulase.
- Efficient inducers are important for cellulase production using fungal strains.
- Integrated multi-omics analyses provide genetic elements for fungal strain development.
- Strategies for metabolic engineering of filamentous fungus were summarized.

GRAPHICAL ABSTRACT



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ABSTRACT

Lignocellulosic biomass (LCB) has been recognized as a valuable carbon source for the sustainable production of biofuels and value-added biochemicals. Crude enzymes produced by fungal cell factories benefit economic LCB degradation. However, high enzyme production cost remains a great challenge. Filamentous fungi have been widely used to produce cellulolytic enzymes. Metabolic engineering of fungi contributes to efficient cellulase production for LCB biorefinery. Here the latest progress in utilizing fungal cell factories for cellulase production was summarized, including developing genome engineering tools to improve the efficiency of fungal cell factories, manipulating promoters, and modulating transcription factors. Multi-omics analysis of fungi contributes to identifying novel genetic elements for enhancing cellulase production. Furthermore, the importance of translation regulation of cellulase production are emphasized. Efficient development of fungal cell factories based on integrative strain engineering would benefit the overall bioconversion efficacy of LCB for sustainable bioproduction.

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1. Introduction

Mitigating humanity's reliance on fossil fuels contributes to achieving long-term environmental sustainability. Bioenergy and biochemicals can be produced using lignocellulosic biomass (LCB), which is the most abundant renewable resource in nature. Due to the easy accessibility, cost-effectiveness, and wide geographic distribution, lignocellulosic biomass is a promising source of biofuels and biochemicals for sustainable production (Saravanan et al., 2022).

LCB mainly consists of cellulose (40–50%), hemicellulose (20–35%), and lignin (15–20%). Among them, cellulose, a straight-chain polysaccharide, is composed of more than 10,000 subunits of glucose residues via β-1,4-glycosidic bonds. Recently, cellulose nanofibrils derived from plants were reported as a rising novel green substrate for biomass conversions (Zhang et al., 2023a; Zhang et al., 2023b). Hemicellulose is a heteropolysaccharide composed of hexoses (glucose, mannose, and galactose), pentoses (xylose and arabinose), uronic acids, and desoxyhexoses (rhamnose and fucose). The amorphous and branched structure of hemicellulose makes it the first biomass fraction to be degraded (Lorenci Woiciechowski et al., 2020). Both cellulose and hemicellulose are degraded into simple sugars such as glucose and xylose by cellulase and hemicellulase, respectively. However, the cost of these enzymes is very high, which limits economic lignocellulosic biorefinery (Kumar Saini et al., 2022).

To address this issue, multiple operation steps have been optimized, including substrate modification, pretreatment, and cellulase production. Modification on plant cell walls provides more selectable and feasible recalcitrance-much-reduced bioenergy crops to enhance enzymatic saccharification (Wang et al., 2021a). Desired pretreatment can also help to generate valuable nanomaterials, such as cellulose nanocarbons and lignin nanoparticles, which facilitate full lignocellulose utilization (Zhang et al., 2023c). Subsequently, microbes are engineered for cellulase production, saccharification, and efficient fermentation for biomass conversion. While random strain breeding can use physical/chemical mutagenesis or laboratory-directed evolution to obtain strains with desired phenotypes under specific conditions (Cho et al., 2022; Grujić et al., 2019), rational engineering on strains to improve cellulase production is still an important strategy (Gao et al., 2022).

In this review, cellulase production using filamentous fungi and its optimization strategies reported in recent years were summarized, and the exploration of optimal inducers for cellulase production was reviewed. The progress of integrated metabolic engineering to improve cellulase production via promoter engineering, transcription factor engineering, and protein secretion and degradation engineering was presented. Furthermore, the current achievements and the promising potential of omics for enhancing cellulase production were presented.

2. High through-put tools boost microbes capable of cellulase production

Microorganisms, including actinomycetes, bacteria, and fungi, possess remarkable abilities to secrete plant-polysaccharide-degrading enzymes that facilitate enzymatic hydrolysis of lignocellulosic biomass in their natural environment (Sethupathy et al., 2021). In terms of optimizing microbial cellulase systems for industrial applications, filamentous fungi are the most preferred due to their strong ability to thrive under aerobic conditions and produce extracellular and heterogeneous proteins with a reasonable structure and ratio. Thus, most cellulase preparations involve the use of filamentous fungi (Paul et al., 2021).

Screening and isolation of highly cellulase-producing microbes can be achieved through high-throughput platforms, such as hydrolysis zone-based high-throughput plating (Zhang et al., 2014), flow cytometry-based ultrahigh throughput screening (uHTS) (Körfer et al., 2016), GFP-fusion coupled fluorescence-activated cell sorting (FACS) for rapid selection of expressible heterologous genes (Wang et al., 2018a), droplet-based microfluidic high-throughput screening (He et al., 2019). For example, a novel high-throughput plating-free method based on flow cytometry in industrial fungi *Myceliophthora thermophila* and *Aspergillus niger* was developed, which could directly screen and isolate the correctly transformed protoplasts, thereby saving time (Yang et al., 2022). Through the use of high-throughput platforms, many filamentous fungi capable of degrading native cellulose have been extensively isolated and identified. In particular, most of the fungal strains identified as highly cellulase-producing belong to the genera *Trichoderma* and *Penicillium*. For example, Li et al. screened and isolated *T. harzianum* strain LZ117 with the highest filter paper cellulase (FPase) production of 0.65 U/mL among 88 fungal strains obtained from Tibetan Plateau soils (Li et al., 2019). Jing et al. obtained the *P. oxalicum* strain Z1-3 that exhibited 2.74 U/mL of FPase production in the presence of wheat bran plus Avicel (Jing et al., 2015).

Additionally, machine learning based on artificial intelligence appears to be an overwhelming priority in characteristic prediction and fermentation optimization. During fermentation, filamentous fungi form different macromorphologies, including pellets, dispersed mycelia, or clumped aggregates associated with final product titers. To quantify the morphology of pelleted and dispersed growth (MPD), an automated image analysis pipeline was developed (Cairns et al., 2019), providing an efficient tool for morphology engineering. In another report, a combination of the new strain *T. harzianum* PWN6 and definitive screening design (DSD) for citric acid biosynthesis was achieved by applying definitive screening design (DSD) and artificial neural network (ANN) (Elsayed et al., 2021). Similar strategies would be helpful to screen and engineer fungal strains to enhance cellulase production, which deserves further exploration.

3. Cocktail of cellulolytic enzymes in filamentous fungi

A well-balanced cocktail of cellulolytic enzymes can accelerate the hydrolysis rate of plant polysaccharides and minimize enzyme requirements (Adsul et al., 2020; Li et al., 2022a). Endoglucanase (EG; EC 3.2.1.4), cellobiohydrolase (CBH; EC 3.2.1.176), and β-glucosidase (BGL; EC 3.2.1.21) are three main types of cellulosic enzymes indispensable for the degradation of cellulose. EGs primarily function in amorphous regions of polymer fibers to hydrolyze randomly β-1,4-glycosidic bonds. Through synergy, CBHs facilitate the release of disaccharide units from the reducing ends of carbohydrate chains without dissociation after each catalytic event. The released oligosaccharides and cellobiose are subsequently hydrolyzed into D-glucose by BGL. According to different structures, these enzymes are grouped into various glycoside hydrolase (GH) families (Lombard et al., 2014). In *T. reesei*, the known EGs belong to GH5, GH7, GH12, and GH45; the known CBHs belong to GH6 (CBH2) and GH7 (CBH1); and the known BGLs belong to GH1 (intracellular enzymes) and GH3 (most are extracellular enzymes).

The cocktail of cellulolytic enzymes secreted by fungal cells is diverse depending on the extracellular inducers, fermentation formats, and induction time. For example, the longer time *T. reesei* strain Rut-C30 was induced by sugarcane bagasse, the more cellulase and xylanase were secreted (Borin et al., 2015). *P. oxalicum* strain GZ-2 produced more cellulase and xylanase on Avicel plus xylan than on sole Avicel (Liao et al., 2014). Furthermore, secretome analysis showed that extracellular cellulase and xylanase levels produced by *P. oxalicum* strain 16 cultivated on rice straw were significantly higher than those on wheat bran, especially CBHs (e.g., CBH1 and CBH2) and EGs (e.g, EG1), comparable to those of *T. reesei* strain Rut-C30 on wheat bran and rice straw. In comparison, *P. oxalicum* strain 16 produced slightly lower CBH but higher EG and BGL than *T. reesei* strain Rut-C30 on either rice straw or wheat bran (Wang et al., 2021c).

In addition to these three main types of cellulosic enzymes, the cellulolytic cocktail also contains cellodextrinase (EC 3.2.1.74), cellobiose phosphorylase (EC 2.4.1.20), cellodextrin phosphorylase (EC 2.4.1.49) and cellobiose epimerase (EC 5.1.3.11) (Sharma et al., 2016).

These non-hydrolytic accessory proteins are required for the optimal function of cellulosic enzymes (Singhania et al., 2021). For example, carbohydrate-binding modules (CBMs) help to enhance cellulase production during the saccharification of lignocellulose, particularly CBM1 derived from cellobiohydrolase I of *T. reesei*, which exhibits a greater impact than bovine serum albumin (BSA), a commonly used accessory protein (Jia et al., 2022). By March of 2023, over 377,332 CBMs, which can be classified into 97 families, with 3948 CBMs still non-classified, have been reported in Carbohydrate-Active enZYmes Database (https://www.cazy.org). In terms of substrate binding, CBM1 is also closely related to the cellulose-binding ability, enzyme activity, and regional selectivity of lytic polysaccharide monooxygenases (LPMOs) (Chalak et al., 2019).

LPMOs are copper-dependent enzymes that facilitate the oxidative breakdown of polysaccharides. This property is highly desirable for industrial applications due to its ability to enhance glycoside hydrolases' enzymatic depolymerization of recalcitrant substrates (Adsul et al., 2020). To enhance the saccharification efficiency, several heterogeneous LPMOs were also identified and incorporated into the *T. reesei* enzyme cocktail for optimization, such as AA16 from *Aspergillus aculeatus* (Filiatrault-Chastel et al., 2019) and TcAA9A from *Thermoascus aurantiacus* (Zhang et al., 2019).

The fungal secretome is also rich in oxidoreductases, including cellobiose dehydrogenases (CDHs) belonging to the AA3_1 family. These enzymes gain electrons by oxidizing cellulose and hemicellulose degradation to lactones, allowing them to interact directly with LPMOs (Sygmund et al., 2012). By binding to the AA8 cytochrome domain, CDHs act as electron donors and H_2O_2 suppliers to reduce the active site copper in LPMOs (Kracher et al., 2020). Although CDHs are not endogenous to *T. reesei*, heterologous expression of CDH from *P. chrysosporium* in *T. reesei* resulted in faster and higher yields than the native host (Wohlschlager et al., 2021). In a recent report, overexpressing the expansin gene *EXLX1* from *Bacillus subtilis* in *T. reesei* boosted cellulase secretion for synergistic enhancement of biomass enzymatic saccharification in corn and *Miscanthus* straws (Xu et al., 2023).

4. Inducers for the biosynthesis of cellulase in filamentous fungi

Cellulase and xylanase biosynthesis of filamentous fungi require induction, e.g., cellulose and its derivates as the inducers. Even though Avicel is commonly used for inducing cellulase production, high cost limits its application in large-scale industrial production. Bioenergy crops such as wheat bran and rice straw have been acknowledged as effective inducers for both *P. oxalicum* and *T. reesei*. Recently, several cost-effective biomasses have been studied to achieve the optimal cellulases and xylanases cocktails, such as characteristic *Miscanthus* (Msi62) de-lignin residue (Liu et al., 2021) and natural rice mutant *Osfc16* (Peng et al., 2022).

Compared to solid complex inducers, soluble ones have higher heat transfer efficiency in the fermenter and lower energy consumption. Additionally, cellulase production can be directly induced in cells with soluble inducers. Lactose, although considered a desirable soluble inducer, produced less cellulase than cellulose (Verma & Kumar, 2022). Sophorose is currently regarded as the most effective inducer of cellulase production for *T. reesei*, but the high cost also confines large-scale applications. A patent from England et al. (2010) showed that a high-concentration sugar mixture could induce the expression of genes under the control of an inducible promoter, indicating that man-made soluble inducers are accessible. Several effective and economical soluble inducers have been explored in cellulase production. For instance, MGD (mixture of glucose and β-disaccharide), containing sophorose from glucose through transglycosylation reaction, has been widely applied in cellulase fermentation (Li et al., 2016; Li et al., 2018; Li et al., 2022b). In a recent work, byproduct sophorose from the stevioside acid hydrolysis process was examined, and the MGS (mixture of glucose and sophorose) was proved to be an effective inducer comparable to MGD (Zhang et al., 2022c).

However, soluble inducers are only limitedly functional in certain species compared to insoluble inducers. For instance, most soluble inducers suitable for *T. reesei*, are not effective for *P. oxalicum*, except cellobiose. Recently, two novel inducers, methylcellulose and 2-hydroxyethyl cellulose, were identified to promote cellulase production by *P. oxalicum* and their effects for enhancing the production of certain cellulase (e.g. FPase) were even higher than Avicel and/or wheat bran to some extent, suggesting that modification of sugar possibly participates in signal process for inducing enzyme biosynthesis (Li et al., 2020).

5. Integrated metabolic engineering strategies to improve cellulase production

Metabolic engineering, which refers to the targeted modification of metabolic pathways in an organism, has become feasible in filamentous fungi due to the development of various methods to control gene expression (Chroumpi et al., 2020). Metabolic engineering aims to manipulate genes involved in a specific step to redirect the metabolic flux towards the desired efficiency or affinity of the encoded enzyme, thereby achieving targeted product synthesis. Strategies for metabolic engineering in *T. reesei* include manipulating factors that regulate carbon catabolite repression (CCR), nutrient sensing, and chromatin remodeling.

Perturbation of gene expression is the most basic and practical approach to functional annotation and pathway manipulation, primarily involving gene knockout or knockdown (Table 1). In general, gene disruption in *T. reesei* is commonly achieved by homology-based DNA recombination (HDR) to knock out target genes. The efficiency of this method is low but can be improved by knocking out *ku70*, *mus53*, and *dmm2*, which are involved in non-homologous end joining (Cai et al., 2022; Chum et al., 2017; Guangtao et al., 2009). Avoiding a gene knockout strategy is advisable in certain circumstances, especially when it involves disrupting critical and recalcitrant genes that are fundamental to viability or multiple essential genes. Improved gene expression modulation strategies, particularly RNAi and CRISPR systems, have been extensively implemented in eukaryotes. On the one hand, based on the principle that Dicer proteins can recognize and cleave double-stranded RNA (Chang et al., 2012), RNAi has also been used for gene silencing in *T. reesei* (Table 2). On the other hand, the successful construction of the CRISPR/Cas9 system in filamentous fungi was first achieved by specific codon optimization and *in vitro* RNA transcription in *T. reesei* (Liu et al., 2015). Subsequently, CRISPR/Cas9-mediated rational engineering of *T. reesei* has been widely applied (Table 3). In recent years, with the development of a genome-editing CRISPR/Cas9 system in the thermophilic fungal *Myceliophthora* species, *M. thermophila* has presented great potential in LCB biorefinery (Zhang et al., 2022a; Zhang et al., 2023e). A comprehensive review on the utilization of CRISPR/Cas technology in *Trichoderma* and other fungi for genetic and metabolic engineering purposes can be referred (Wang et al., 2022a), including gene silencing, mutagenesis, regulation, and tagging.

To improve cellulase production, rational strategies based on efficient engineering toolboxes can overcome bottlenecks and improve metabolite yields in complex fermentation environments (Table 4). Revising the metabolic processes of cellulase production usually involves integrating different engineering toolboxes as harmonious modules rather than completely tearing them apart. Here, promoter engineering, transcription factor engineering, and metabolic engineering and their applications in improving the productivity of cellulase enzymes were reviewed (Fig. 1).

5.1. Promoter engineering

Promoters, defined as DNA sequences that can facilitate the binding

Table 1
Functional proteins and regulatory elements in *T. reesei*.

Name	Functional annotation	Effects of on cellulase production	Reference
Tre108642	Unknown protein	Mutation of *Tre108642* leads to 83.7% reduced production of cellulase	(Liu et al., 2019)
Tre56839	Zinc-dependent alcohol dehydrogenase	Mutation of *Tre56839* leads to 70.17% reduced production of cellulase	
Tre108784	CENPB-type Alcohol dehydrogenase GroES	Mutation of *Tre108784* leads to 53.4% reduced production of cellulase	
Tre55868	Serine/threonine phosphatase	Mutation of *Tre55868* leads to 66.3% increased production of cellulase	
Tre111216	Class II Histone H3 methyltransferase	Mutation of *Tre111216* leads to 66.3% increased production of cellulase	
Tre3529	Alpha and gamma adaptin binding protein p34	Mutation of *Tre3529* leads to 66.3% increased production of cellulase	
Tre80339	Apolipophorin-III and similar insect proteins	Mutation of *Tre80339* leads to 66.3% increased production of cellulase	
Tre81043	Zinc fnger, TFIIS-type	Mutation of *Tre81043* leads to 66.3% increased production of cellulase	
TrISW1	Chromatin Remodeler	Deletion of *TrISW1* abolished (hemi) cellulase gene expression	(Cao et al., 2021)
Lac1	MFS sugar transporters	Deletion of Lac1 almost abolished cellulase production	(Wang et al., 2022c)
Vib1	P53-like regulator	Deletion of Vib1 almost abolished cellulase production, while overexpression enhanced cellulase production by 2 fold	(Sun et al., 2022b; Zhang et al., 2018b)
ZafA	Zinc-responsive transcription factor	Deletion of zafA enhanced 160.4% in pNPCase 70.4% CMCase activities	(Li et al., 2023b)
Ace4	Transcription factors	Overexpression of ace4 increased cellulase production by approximately 22%	(Chen et al., 2021)

of transcription factors and the initiation of transcription, are essential for controlling gene expression in synthetic biology applications. Promoter engineering, as a DNA-based strategy, provides precise gene expression controls in specific conditions at transcription levels, which would further maximize product formation. Strategies of promoter engineering in ascomycete fungi are now involved in several aspects, including construction of promoter library (Sun et al., 2012), the modification of promoter architecture (Blazeck & Alper, 2013), constructions of inducible promoter systems (Kluge et al., 2018), and rational design of hybrid promoters (Deaner & Alper, 2018).

Promoters can be broadly classified into constitutive (or auto inducible) and tunable (or inducible) promoters. Constitutive promoters have no on/off option and are independent of environmental factors, which is considered a desirable alternative. However, as the uncontrollable property is not entirely feasible for practical production, the use of constitutive promoters is not as widespread as that of tunable promoters. Compared to constitutive promoters, tunable promoters are much more flexible in responding to the presence or absence of biotic or abiotic factors such as sugars, amino acids, vitamins, metals, light, or temperature. In addition, the strength of these promoters can be fine-tuned by the number of stimuli applied. Both the constitutive and tunable promoters used in *T. reesei* have recently been described in the review (Adnan et al., 2022).

The endogenous promoters are limited in achieving the maximum transcription levels within an organism. To overcome this issue, several methods have been conducted: (i) introduction of multiple copies of core promoters to increase expression, such as cassette duplication to enhance EG production (Karhunen et al., 1993); (ii) Constructing chimeric promoters synthetically by combining different enzymes, such as the *xyn1-xyn3* chimeric promoter to enhance saccharification ability (Hirasawa et al., 2018) and novel hybrid promoter Pcc to co-overexpress BGLA and EG2 (Wang et al., 2022b); (iii) Modification of repressor binding sites to those of activators, such as replacing the binding sites of the transcriptional repressor ACE1 with activator ones (Sun et al., 2020). These manipulations have significantly increased the efficiency of cellulase gene expression in *T. reesei*, and might be also effective in other fungal strains.

5.2. Engineering transcription factors

Genetic engineering manipulation of transcription factors is a common practice in eukaryotes. The production of cellulose enzymes is highly regulated at the transcriptional level, which can be manipulated to optimize enzyme productivity. Several strategies have been implemented to enhance cellulosic enzyme production based on the distinct properties of different transcription factors.

One is to disrupt repressive transcription factors or overexpress active regulators by inserting specific engineered expression cassettes. For example, to compensate suboptimal ratio of the cellulase cocktail produced by *T. reesei*, a novel *bgl* gene expression cassette was inserted into the locus of repressor Ace1, thereby increasing the Bgl production (Xia et al., 2018). Similarly, to maximize the advantages of Eg1 (Cel7B) for more efficient conversion of various substrates, *eg1* was overexpressed at the *ace1* locus to relieve the repression during cellulase biosynthesis (Meng et al., 2018). In another report, a strategy of inserting the *xyr1* overexpression cassette into the *ace1* locus was developed to upregulate *xyr1* and downregulate *ace1* (Yan et al., 2021). In *P. oxalicum*, an overexpression cassette of *eEF1A* that encodes translational elongation factor 1A was used to replace the locus of transcriptional repressor gene *cxrC*, thereby considerably increasing the

Table 2
RNAi applied for metabolic engineering in *T. reesei*.

Strains	Plasmid skeleton	Promoter	Terminator	Targeted gene	Ref.
T. reesei N10	pSKpyr4	P*cbh1*	T*chb2*	*lip*	(Qin et al., 2012)
T. reesei QM9414	pPtef1-hp	P*trpC*	T*trpC*	*xyiH*	(Hong et al., 2014)
T. reesei DES-15	pCAMBIA1300-1	P*rp2* P*trpC*	–	*gfp* inserted as reporter (1) *rhoA* (2) *cla4* (3) *ras2*	(He et al., 2015)
T. reesei QM9414	pMD19T-*hph*	P*tcu1*	T*cel6a*	*fab1*	(Wang et al., 2018b)
T. reesei QM9414	pMD19T-*hph*	P*tcu1*	T*cel6a*	*rxe1*	(Wang et al., 2019b)
T. reesei SUS2	pAPA	P*pdc1* P*eno1*	–	*Trcot1*	(Gao et al., 2020)
T. reesei QM9414	pRLMex30	P*tcu1*	T*cel6a*	*hph*	(Wang et al., 2021b)

Table 3
CRISPR system applied for metabolic engineering in *T. reesei*.

Species	*Cas9* expression, promoter, marker	promoter for driving gRNA	Delivery method	Editing method	Application, efficiency	Ref.
T. reesei Rut-C30, *T. reesei* QM6a	Codon-optimized Cas9 introduced into the genome, P*pdc*/P*cbh1*, *ura5*	T7 promoter	AMT	NHEJ /HDR	Homologous recombination frequency⩾ 93%	(Liu et al., 2015)
T. reesei TU-6	In vitro constructed Cas9/gRNA complex introduced into the genome, P*pdc1*, *pyr4*	T7 promoter	PMT	NHEJ	–	(Hao & Su, 2019)
T. reesei QM6a	Codon-optimized Cas9 introduced into the genome, P*pdc*/P*cbh1*, *ura5*	U6 promoter identified in *T. reesei*	AMT	NHEJ	Mutation efficiency increased to 8% −10% with purified conidia	(Wu et al., 2020)
T. reesei Rut-C30	Construction of plasmid pTrCas9gRNA1 based on Codon-optimized Cas9, P*pdc1*/P*eno1*, *hph*	–	PMT	NHEJ/ HDR	The genomic integration of donor cassettes ranging from 10 to 16%	(Fonseca et al., 2020)
T. reesei QP4	Construction of plasmid	5S rRNA promoter	PMT	HDR	5S rRNA promoter raised from 6.7% (native) to 36.7% (heterologous)	(Wang et al., 2021d)

Abbreviations: AMT, *Agrobacterium tumefaciens*-mediated transformation; PMT, polyethylene glycol (PEG)-mediated chemical transformation.

Table 4
T. reesei strains manipulated for enhancing enzyme productivity.

Strain code	Gene manipulation	Fermentation	Products	Ref.
Rut-C30	*Trhmg*1 overexpression	Cellulose in flask	9.46 mg/L β-carotene 0.14 U/L *p*NPCase production	(Li et al., 2023a)
		MGDS 30 L fermenter	22.33 IU/mL FPase production 286.63 mg/L β-carotene	
QM6a	*rsr1* deletion	Avicel in flask	Increased by approximately 100% in pNPCase, CMCase, and FPase activities	(Li et al., 2023c)
TU-6	*AN-PEP* heterogeneous overexpression	Avicel in flask	Up to 16.148 U/mL prolyl endopeptidase production (highest titer reported to date)	(Liu et al., 2023)
QM9414	Fusing the DNA binding domain of Xyr1 to the transactivation domain of Tmac1	Glucose in flask	1.2-fold increase in FPase production, and 8.2-fold increase in xylanase activity, with saccharification efficiency 60% increase (equal broth volume), compared to QM9414	(Lv et al., 2023)
QM53	BglA and Eg2 co-overexpression by Pcc promoter	CPM in flask	Cellulase activity increased by 178.1% and 156.5%	(Wang et al., 2022b)
QM9414 Δ*pyr4*	Simultaneous deletion of *eg1*, *eg2*, *cbh1*, and *cbh2* with *xyr1* overexpression	Lactose in flask	Up to 8.06 U/mL α-arabinofuranosidase activity increased by 8-fold, and saccharification assay with 5% pretreated corn fiber releasing 1.6-fold and 2.6-fold higher yield of arabinose and xylose	(Zhang et al., 2022b)
QM9414 Δ*pyr4*	*gat1* deletion	Avicel in flask	Increased by approximately 40% in xylanase activity	(Xu et al., 2022)
QP4	*gul1* deletion	Cellulose in flask	3.38 U/mL FPase production increased by 22%	(Zhao et al., 2021)
NG14	*ace3* truncation	MGDS 30 L fermenter	102.63 IU/mL FPase production	(Chen et al., 2020)
T. reesei QM9414	Xyr1 overexpreesion at the *pyr4* gene locus and reintroduction *pyr4* back with	Avicel in flask	up to 5 IU/mL Fpase, and saccharification assay with 5% pretreated corncob residues releasing 85% higher glucose	(Zheng et al., 2020)
QM9414 Δ*mus53*	Simultaneous deletion of protease coding genes tre81070, tre120998, and *tre123234*	CPM in flask	2.08 U/ml FPase production increased by 6-fold, and β-glucosidase (BGL), CBH, EG, and xylanase activities exhibited 150%, 75%, 65% and 6% higher activities	(Qian et al., 2019)
Rut-C30	Mutated Xyr1 (XMG) overexpression	Lactose in flask	1.8 U/mL xylanase production 0.146 U/mL *p*NPCase production	(Zhang et al., 2018a)
		Avicel in flask	2.9 U/mL xylanase production 0.328 U/mL *p*NPCase production	

Abbreviations: MGDS, mixture of glucose and β-disaccharides; CPM, cellulase production medium.

production of cellulase (Zhao et al., 2022). Also, combinatorial engineering of three activators, ClrB, XlnR, and AraR, generated an on-site hyperproduction strain of lignocellulosic enzyme, with more fermentable sugars released from corn fiber than the parent strain (Gao et al., 2021). This type of strategy would not only achieve improvement of cellulase composition intentionally but substantial enhancement of cellulase production.

In addition, constructing chimeric transcription factors is a promising strategy for regulating cellulase synthesis. This involves fusing zinc finger domains of transcription factors with effector domains derived from well-studied activators or repressors. Specific artificial transcription factors have been constructed to enhance cellulase production. For example, a novel chimeric transcription activator was designed by fusing the CRE1 DNA-binding domain and the XYR1 DNA-binding domain with an effector domain, resulting in a marked improvement in cellulase production (Zhang et al., 2017). Four chimeric transcription factors were constructed with fused zinc finger regions of CRE1 and ACE1, alleviating the repression of both factors by competing for CRE1 and ACE1 binding sites (Wang et al., 2019a). It was demonstrated that by replacing the C-terminus of Cre1 in *T. reesei* with that of CreA from *P. oxalicum*, the Cre1 chimera significantly alleviated the CCR effect and greatly enhanced cellulase production in the presence of glucose (Han et al., 2020). These artificial transcription factors, which can be used flexibly in different combinations, are a great technical tool for adjusting the ratios of cellulase cocktails for different purposes.

Another approach is to use an artificial zinc finger protein (AZFP) library to identify novel genes encoding cellulolytic enzymes or transcription factors. Another approach is to use an artificial zinc finger protein (AZFP) library to identify novel genes encoding cellulolytic enzymes or transcription factors. Zinc finger domains in transcription

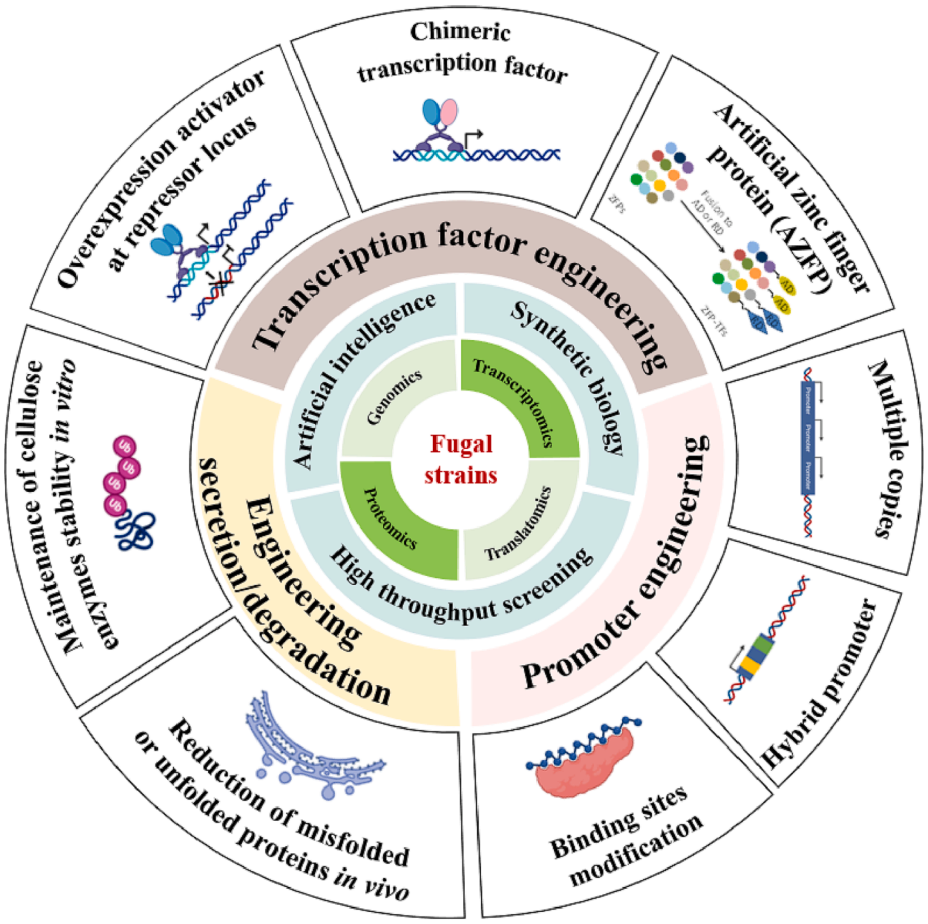

Fig. 1. Strategies for genetic manipulation of fungal strains to produce cellulase.

factors confer distinct functions through variations in key amino acid residues, providing a vast array of combination possibilities for specific recognition at promoter regions of target genes. By exploiting the modular architecture of zinc finger proteins (ZFPs), the AZFP library to enhance the cellulase production of *T. reesei* Rut-C30 was constructe (Zhang et al., 2016). A mutant AZFP-U5 was selected from the plasmid library due to its high protein secretion and cellulase production (Zhang et al., 2020). Subsequently, enhanced cellulase production was achieved by optimizing AZFP by substituting its Gal4 activation domain with the endogenous activation domain of the transcriptional activator Xyr1 (Meng et al., 2020).

5.3. Engineering protein folding, secretion and degradation

Reducing misfolded or unfolded proteins *in vivo* before secretion and maintaining the stability of cellulolytic enzymes *in vitro* after secretion is important for enzyme activities. Misfolded proteins are potentially toxic proteins in the lumen and membrane of the endoplasmic reticulum (ER), and endoplasmic reticulum-associated degradation (ERAD) is known to be important for protein secretion by removing the misfolded proteins (Krshnan et al., 2022). Studies on engineering protein folding have been proven to promote secretive protein production in filamentous fungi (Sun et al., 2022a; Wu et al., 2017). In *T. reesei*, it was demonstrated that a defective ERAD pathway negatively affects fungal growth and cellulase secretion (Yao et al., 2023), providing new insight into the cellulase secretion mechanism in *T. reesei*. Additionally, heterologous overexpression of *lacA* of *Trametes* sp. AH28-2 in *T. reesei* was found to strongly activate ERAD and unfolded protein response (UPR) (Zhang et al., 2023d). At the same time, the activation diminished when the *lacA* gene was integrated into the *cbh1* locus, indicating the reduction of secretion pressure by deleting endogenous genes is an efficient strategy for the secretion of heterologous proteins.

From an industrial point of view, the stability and reusability of enzymes are essential. In addition to improving the production of cellulosic enzymes, it is crucial to address the issue of proteolytic degradation, especially for heterologous proteins that are more susceptible to degradation. The expression of proteases was regulated by different nitrogen and carbon sources in the growth environment, which helps consciously control unwanted proteolysis to establish cell factories for specific production accumulation (Sun et al., 2021). Given the abundance of proteases in fungal genomes, deleting protease-encoding genes or related transcription factors are rational strategies to enhance cellulosic enzymes and heterologous production (Lübeck & Lübeck, 2022; Sun et al., 2021). Recently, a *T. reesei* chassis strain with reduced extracellular protease activity and high production of heterologous bacterial XYL7 was successfully established using CRISPR/Cas9 (Chai et al., 2022).

6. Omics approaches in the application of cellulase production enhancement

Advances in next-generation sequencing, omics technologies, and bioinformatics have brought microbial research into a new era. Genomic, transcriptomic, and proteomic analyses have provided abundant data for optimizing cellulase production, which were summarized below.

6.1. Genomic analysis on cellulase production

Since the genome of *T. reesei* was sequenced (Le Crom et al., 2009),

omics analyses have played a crucial role not only in identifying the specificity of the fungus among different fungi (Gupta et al., 2016) and providing insights into the evolution of mycoparasites (Kubicek et al., 2019), but also in investigating the mechanisms behind cellulase enhancement through multiple comparative omics.

A comparative genomic screen was conducted between the *T. reesei* cellulase-hyperproducing strain SS-II and Rut-C30, both derived from the *T. reesei* NG14 strain, contributing to creating a gene library that can be used to investigate the regulation of cellulase production (Liu et al., 2019). In addition, a novel transcriptional activator ACE4 was discovered through comparative genomic screening (Chen et al., 2021), the deletion of which significantly affected the expression of four key cellulase genes and the transcriptional activator gene *ace3*. In another report, the genome of *T. asperellum* ND-1 was compared with *T. reesei* QM6a and other fungi and found that ND-1 contained a unique enzymatic composition with higher hemicellulase (particularly xylanase) and cellulase activities, which could provide a promising platform for industrial ND-1 design (Zheng et al., 2022). Similarly, comparative genome analyses of the *P. oxalicum* wild-type strain HP7-1 and its derived mutant EU2106 were performed (Zhao et al., 2016), and two novel regulatory genes, *POX02484* and *POX08522*, were identified, which can be used to modulate cellulase production.

6.2. Transcriptomics studies on cellulase production

Compared to the stable nature of the genome, the transcriptome exhibits temporal and spatial dynamics (Hesham et al., 2020). A time-course experimental report was conducted to monitor the degradation of pretreated bagasse through comparative transcriptomics between *A. niger* and *T. reesei*, which provided a rational design for enzyme cocktails using different strategies for biomass degradation (Borin et al., 2017). Transcriptomic analyses were performed to compare cellulase production in *T. reesei* Rut-C30 induced by β-disaccharide and lactose, providing evidence for the use of β-disaccharide as an inducer and practical strategies for strain engineering (Li et al., 2021). In *P. oxalicum*, weighted gene co-expression network analysis was used to elucidate carbon source-specific and time-course transcriptional patterns and then found three novel regulatory genes for cellulase gene expression in combination with molecular genetic analysis (Li et al., 2020).

6.3. Proteomic analysis on cellulase production

Comparative proteomics is an essential tool as it accurately reflects the gene expression level. As a part of proteome, secretory proteins comprise almost 30% of the proteome and are essential for almost all physiological, developmental, and pathological processes. Comparative secretomics is a rational testing tool for comprehensively characterizing extracellular and intracellular proteomes using quantitative protein detection methods such as mass spectrometry (MS). Via comparative secretomes, the influence of different substrates on cellulase production was investigated (Wang et al., 2021c) and it was found that wheat bran promoted more balanced GHs production in *P. oxalicum16* and *T. reesei* RUT-C30 compared to rice straw.

7. Future prospects

Improving the productivity of cellulosic enzymes is an inevitable link in the biorefinery industry. Although an increasing number of microbial strains possessing cellulase hyper-productivity have been screened, reconstruction engineering is still required for industrial applications. *T. reesei*, as a relatively mature model for lignocellulosic biomass degradation for decades, not only serves as a promising important industrial strain and provides rational engineering references for other organisms. As the theoretical maximum yield approaches, further increases will be more difficult. Therefore, efforts to improve production strains have been directed toward more rational and systematic engineering strategies. Promoter engineering, transcription factor engineering, and protein secretion/degradation engineering have achieved high percentages of improvements in *T. reesei*, involving a variety of methods such as overexpression of transcriptional activators, disruption of transcriptional repressors, increasing gene copy number, knock-in or knock-out of targeted genes by CRISPR system, omics analysis, etc. Through improved engineering, the precise tuning and balancing of cellular metabolism should be achieved to increase yields and reduce by-product formation, and robust microbial bioproduction performance can be maintained during large-scale fermentation.

To date, most studies on the regulation of cellulase production in *T. reesei* have focused mainly on the transcriptional and secretomic levels. However, the secretion of proteins expressed in *T. reesei* is a complex process involving multiple regulatory steps, including transcription, translation, folding, post-translational modification, translocation, quality control, and proteolysis, which determines a possible unequal abundance between mRNAs and proteins. Therefore, how the black box of the translatome works between the transcriptome and the secretome or proteome is a tricky but important question to unravel the regulation of the productivity of cellulosic enzymes so that more efficient rational strategies can be proposed to support the optimization of the metabolic mechanism.

Despite studies in genome, transcriptome, proteome, and secretome, so far, no studies of filamentous fungi have been reported at the translation level to reveal regulation of cellulase production or secretion. Studies on yeast have shown that most changes in protein abundance can be explained by mRNA abundance, but the correlation between protein and mRNA is dynamic throughout the cell cycle or under different stresses (Lahtvee et al., 2017). It will be interesting to simultaneously study transcriptome and proteome changes during cellulase production. However, so far, no relevant results have been reported.

Compared to transcriptome with broad data, translatome data would provide much more selective data, precisely focused on active mRNA populations, which could help to identify the core regulators under translational control (Meteignier et al., 2017; Zupanic et al., 2014). To date, for studies on the mechanism of cellulase production, there is still a lack of a systematic network to monitor variations at the DNA-RNA-protein level.

Ribosome profiling (Ribo-seq) has been developed for translatome studies in mammalian cells (Ingolia et al., 2012). Unlike other techniques, ribosome profiling measures the translated mRNA bound by ribosomes, which can directly determine the actual RNA region being translated and analyze different aspects of translation as well as the rate of translation (Sharma et al., 2021). In addition to ribosome profiling, polysomal profiling, and ribosome affinity purification are applied to translatome analysis in eukaryotes (Sharma et al., 2017; Zhu et al., 2023). Novel technologies such as surface sensing of translation (SUnSET), a non-radioactive fluorescence-activated cell sorting-based assay, are used to monitor and quantify global protein synthesis (Jeelani & Nozaki, 2021). Translatome and protein translation studies on cellulase production may provide novel targets to improve cellulase production, which deserves exploration in various fungal species. Integrative strain engineering using targes identified from various aspects of omics analyses (Fig. 2) is prospected to develop efficient fungal cell factories for cellulase production.

8. Conclusions

Selection of robust fungal strains and integration of multi-omic analyses data to engineer the host strains will benefit the development of fungal cell factories with improved cellulase production. Various tools can be used for fungal strain engineering, including genome engineering based on the CRISPR-Cas9 genome editing system, promoter and transcription factor engineering, and signal transduction-pathway modulation. Combining synthetic biology and artificial intelligence will promote the efficiency of fungal metabolic engineering to improve

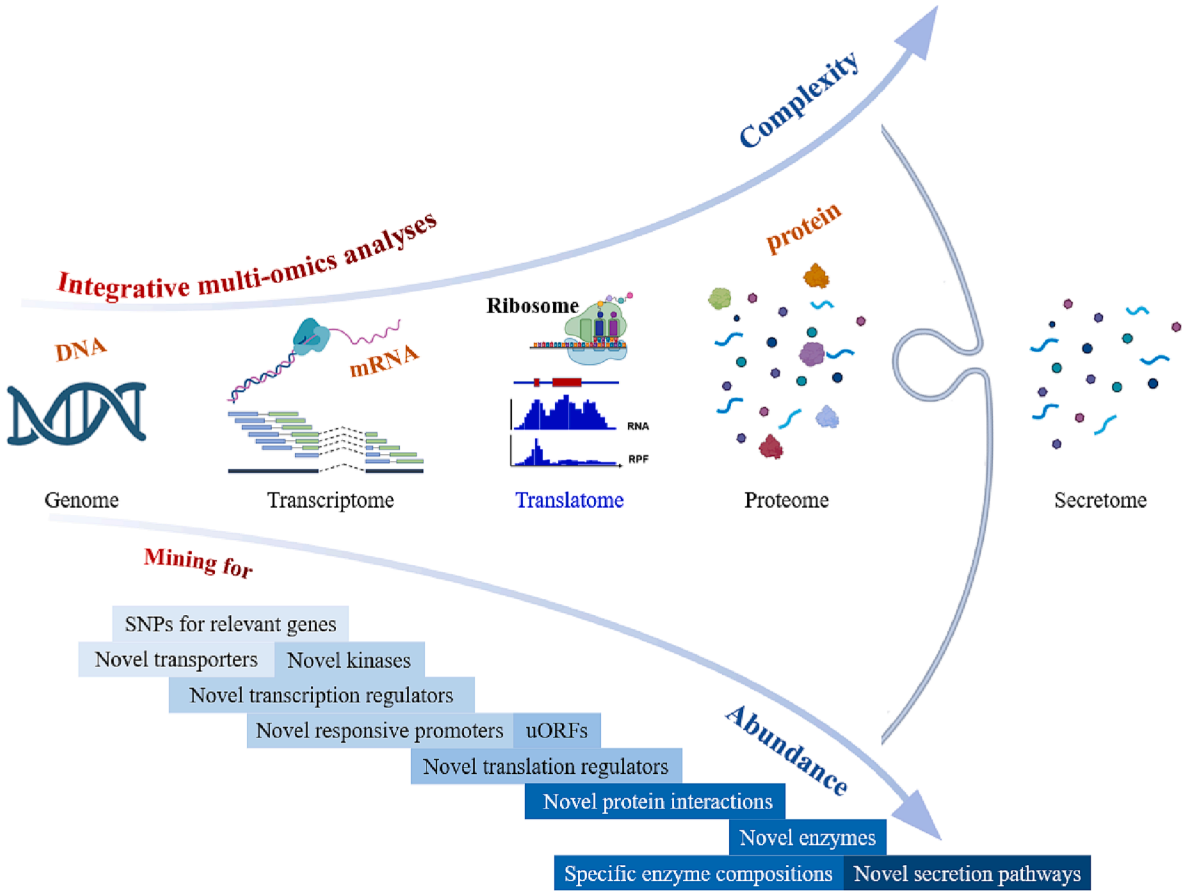

Fig. 2. Multi-omics analysis at various steps for identification of genetic engineering targets to increase cellulase production.

cellulase production. Low-cost crude enzymes produced by optimized fungal cell factories would provide a basis for efficient LCB biorefinery.

CRediT authorship contribution statement

Jie Yang: Investigation, Writing – original draft, Writing – review & editing. **Hou-Ru Yue:** Writing – review & editing. **Li-Ya Pan:** Writing – review & editing. **Jia-Xun Feng:** Supervision, Funding acquisition, Project administration. **Shuai Zhao:** Supervision, Funding acquisition. **Surisa Suwannarangsee:** Supervision. **Verawat Champreda:** Supervision. **Chen-Guang Liu:** Supervision. **Xin-Qing Zhao:** Writing – review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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