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REVIEW

Tailoring in fungi for next generation cellulase production with special reference to CRISPR/CAS system

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Abstract

Cellulose is the utmost plenteous source of biopolymer in our earth, and fungi are the most efficient and ubiquitous organism in degrading the cellulosic biomass by synthesizing cellulases. Tailoring through genetic manipulation has played a substantial role in constructing novel fungal strains towards improved cellulase production of desired traits. However, the traditional methods of genetic manipulation of fungi are time-consuming and tedious. With the availability of the full-genome sequences of several industrially relevant filamentous fungi, CRISPR-CAS (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) technology has come into the focus for the proficient development of manipulated strains of filamentous fungi. This review summarizes the mode of action of cellulases, transcription level regulation for cellulase expression, various traditional strategies of genetic manipulation with CRISPR-CAS technology to develop modified fungal strains for a preferred level of cellulase production, and the futuristic trend in this arena of research.

Keywords Cellulose · Cellulase · Fungi · Genetic manipulation · CRISPR · CAS

Introduction

Lignocellulosic biomass covers the earth's largest reservoir of inexhaustible resources, representing about 1.3 billion tons per year [1, 2]. Cellulose comprises a significant fraction of lignocellulosic biomass, sharing up to 50%, and globally it is the most plentiful polysaccharide. Cellulose is a linear polymer consisting of β-1,4-linked D-glucose residues [3]. Hydrogen bonds tightly hold these glucose chains to form insoluble fibrous materials. Structurally, a two-phase model involving crystalline and amorphous phases intermittent with a chain of semi-crystalline structures makes it challenging to utilize active carbohydrate degrading enzymes [4]. As a polysaccharide, cellulose served as a significant milestone of bioenergy production (methane, ethanol, and biofuels), making it a hot topic of renewable bioenergy research [5]. The most efficient, eco-friendly, and sustainable biomass hydrolysis (cellulosic fraction) method is the enzymatic saccharification by cellulase. More than two centuries ago, Anselme Payne was the first to discover and isolate these incredible enzymes from plants [6].

Enzymatic hydrolysis of cellulose required collaborative action of three enzymes: endoglucanase or CMCase (EG) (EC 3.2.1.4), which cleaves internal β-1,4-glucosidic bonds randomly; exoglucanase or cellobiohydrolase (CBH) (EC.3.2.1.91), which cleaves off cellobiose units from the ends and β-glucosidase (BGL) (EC.3.2.1.21), which transforms cellobiose and cellodextrins into glucose [7, 8]. For cellulase production, fungi are preferable to bacteria because bacterial cellulase usually lacks one of the three cellulolytic activities (exoglucanase activity), downstream processing of fungal cellulase is much easier than bacterial cellulases, and the activity of fungal cellulase is far greater than that of the bacterial cellulase [6]. Fungi is regarded as one of the earliest eukaryotic life appearing on the earth, confirmed by the fossils dating (0.45–0.46 billion years ago) [138]. The ecological significance of fungi is due to their potentiality as dominant decomposers in the ecosystem. Filamentous fungi are major cellulase producers, including *Trichoderma reesei*, *Aspergillus niger*, *Neurospora crassa*, and *Penicillium*

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oxalicum, gain special attention and have been rigorously studied [9].

Due to a diverse range of industrial applications like pulp and paper, bakery, textile, food processing, agricultural, biotechnology, etc., cellulases have a massive demand among the commercialized industrial enzymes [10]. They constitute ~3/4 of the need for total enzymes required in biorefineries, pulp and paper, textile, and food/feed industries [11]. According to Markets and Markets report, 2020 (https://www.marketsandmarkets.com) the global enzyme market is projected to grow from 10 billion US dollar in 2019 to 14.7 billion US dollar in 2025, and cellulase is a frontrunner candidate of the global enzyme market. Cellulases' market value emerging at 5.5% expected compound annual growth rate during 2018–2025 and will reach 2300 million US dollar by 2025 [12]. However, the high expenditure of cellulase production is one of the bottlenecks for the industrialization of lignocellulosic bioconversion. Researches on cellulase production using wild-type strains of fungi have been done extensively. Still, the main drawbacks of the wild-type strains are the unavailability of hyper-producing efficient strain, incapability of large-scale production, and incompetence to tolerate industrial operations' extreme conditions [147]. The cellulases from *Aspergillus* sp. usually have high BGL levels and lower EG activity; on the other side, *Trichoderma* has high EG and CBH activity but lowers BGL level, so there is some extent of limitation in efficient cellulose hydrolysis [13].

Genetic modification of fungal strains through traditional methods or CRISPR/CAS system enables the synthesis of cellulase with enhanced catalytic properties that can manage the demand of industrial sectors [14]. However, simultaneous operation at multiple target sites is the major bottleneck of traditional genome editing tools. The CRISPR-CAS-mediated double-stranded breaks are repaired by the process of nonhomologous end-joining, differ from the homologous recombination where long sequence homology is obligatory. The cutting-edge genome-editing approaches such as split marker technique, transcription activator-like effector nucleases (TALENS), and zinc finger nucleases (ZFNs) also have some complications like time-taking multiple rounds of marker selection and difficulties in the preparation of specific DNA binding proteins along with inadequacy in simultaneous editing of multiple targets. The CRISPR-CAS system gets over from such difficulties. Besides, multiplexed CRISPR strategy (a modified version of the CRISPR-CAS system) allows synchronized activation or repression of genes during the editing of metabolic pathways [15, 16].

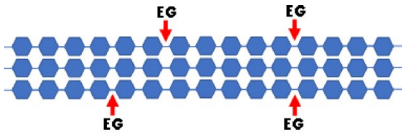
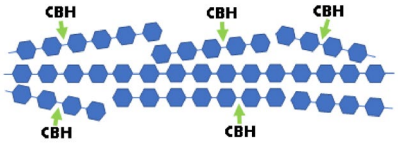
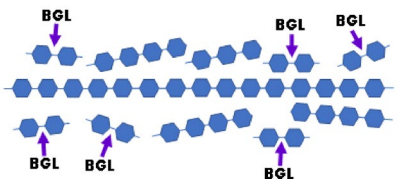
Different attributes of enzyme viz. enantioselectivity, stereospecificity, substrate specificity, enzyme activity, enzyme stability, and tolerance have been ameliorated through genetic manipulations [17]. For the production food grade cellulases, manufacturers should be conscious about the safety issues. By deploying this approach, it is possible to produce cellulase in a non-pathogenic strain by introducing the cellulase gene of the pathogenic one, which is suitable for food safety. In contemporary times, innovative methods and tools at the molecular level have continuously flourished, which intensified the proficiency of genetic manipulation of filamentous fungi for the hyper-production of cellulase. In this deliberation, we shall discuss a brief account of the biochemical, genetic, and molecular nature of cellulase and different genetic manipulation approaches applicable in fungi with special emphasis on CRISPER/CAS technology for improved cellulase production.

Functional properties of cellulases

Cellulose is a hydrophilic, water-insoluble polymer comprising repetitive units of D-glucose linked together by β-1,4-glycosidic bonds. Cellulases belong to the glycoside hydrolases (GHs) class, which degrade cellulose into cellodextrin, cellobiose, and glucose. In general, they comprise a catalytic domain (CD) that breaks down the glycoside bond and carbohydrate-binding module (CBM) that sets the targets of CD to their polysaccharide substrate. Three different cellulolytic enzymes namely CBH, EG and BGL acts synergistically and hydrolyzes cellulose fibre (Table 1). The EG acts randomly on the cellulose fibre in the internal site exposes reducing and non-reducing ends. The CBH acts on these reducing and non-reducing ends to produce cello-oligosaccharides and cellobiose moieties. Finally, the BGL breaks cellobiose and releases glucose (Singhania et al. 149).

Hydrolysis of the cellulose crystalline structure is assisted by lytic polysaccharide monooxygenase (LPMOs), non-hydrolytic proteins, triggering nicks on cellulose fibers to quicken the endoglucanase action. The LPMOs are oxidative enzymes, require an external electron donor (in this case lignin) and molecular oxygen or hydrogen peroxide for their catalytic activity, and elevate the hydrolytic potentiality of glycoside hydrolases (GHs) in the course of the depolymerization of recalcitrant polysaccharides, such as cellulosic biomass. [18]. Commercial cellulase preparations currently include LPMOs as their presence reasonably minimizes enzyme loading [19]. Expansins is a kind of proteins does not have any hydrolytic activity, instead, it is hypothesized to act as a zipper to untie the cross-links between the cellulose microfibrils by relaxing the firmly adhered chains, led to increasing cellulose exposure to cellulases [20]. Expansin-like proteins have been recognized in both bacteria and fungi. Baker et al. [21] reported a mixture of expansin and cellulases from *T. reesei*, resulting in a 13% enrichment in cellulose conversion rate in terms of sugar released from the pretreated yellow poplar sawdust in comparison to cellulase only. Combining recombinant

Table 1 Cellulolytic enzymes, their catalytic pattern, and the cellulase genes of different fungal species Modified version reported by [20, 23, 96]

Enzymes	EC no.	Pattern of cleavage	Gene abbreviation	Number of genes/cellulases in different fungal species
Endoglucanase	EC 3.2.1.4		*egl*	*Aspergillus niger:2* *Myceliophthora thermophile:8* *Nerospora crassa:4* *Penicillium decumbens:11* *Trichoderma reesei:8*
Cellobiohydrolase	EC.3.2.1.91		*cbh*	*Aspergillus niger:2* *Myceliophthora thermophile:7* *Nerospora crassa:3* *Penicillium decumbens:3* *Trichoderma reesei:2*
β-glucosidase	EC.3.2.1.21		*bgl*	*Aspergillus niger:5* *Myceliophthora thermophile:9* *Nerospora crassa:1* *Penicillium decumbens:11* *Trichoderma reesei:7*

EXLX1 protein (a member of the expansin superfamily) and commercial *T. reesei* cellulases led to enhancement of cellulolytic activity [20]. Another protein known as swollenin (expansins like proteins, belonging to glycosyl hydrolase family 45) isolated from *T. reesei*, participates in the deconstruction of the plant cell wall by breaking the hydrogen bond between the microcrystalline cellulose, facilitating further cellulase hydrolysis. Besides, swollenin exhibits glycoside hydrolase activity [20, 22]. Therefore, all these enzymes or proteins act together in a perfect synergism to cleave such a recalcitrant cellulose crystalline structure.

Molecular mechanisms behind the cellulase expression

Plant cell wall polysaccharides' efficient breakdown requires various cellulases to be secreted in massive amounts and a complex regulatory system to control cellulase-related genes. These genes are regulated by several transcription factors (TFs) (Table 2), which are the potential targets for the genetic manipulation of cellulase production. Moreover, efficient genetic engineering of a specific preferred gene in an organism requires detailed knowledge about its genome's genetic architecture and the regulatory mechanism behind that particular gene of interest. A maximum number of TFs participated in such regulatory mechanisms (Fig. 1), particularly from the Zn2Cys6 zinc binuclear cluster family, specifically in fungi [16].

Genetic manipulation for intensified cellulase production

The ability of dominant fungal genera of cellulases producers like *Trichoderma, Aspergillus, Neurospora*, *Myceliopthora*, and *Penicillium* spp. for cellulosic biomass degradation has been practiced formerly. The development of

Table 2 Transcription factors involved in cellulase expression

Transcription factors	Function	References
Tmk1, Tmk2, and Tmk3	In *T. reesei*, it causes phosphorylation and activation of TFs liable for cellulase (cbh1, cbh2, egl1, egl2, and bgl1) production. Removal of *tmk3* down regulated the transcription level expression of cbh1, cbh2, egl1, egl2, and bgl1	[97]
XlnR/ Xyr1	XlnR (xylanase regulator), primarily in *Aspergillus sp.*, and Xyr1 (xylanase regulator 1), primarily in *T. reesei* are the prime transcriptional activators of the genes encoding cellulase (*cbh1, cbh2, egl1*, and *bgl1*) enzymes	[98]
RXE1	It (RXE1 orthologs in *Aspergillus chrysogenum, Beauveria bassiana*, and *Fusarium oxysporum* are designated BRLA) regulate cellulase gene expression, exhibits intense binding activity to the *xyr1* promoter	[99]
Rce1	It acts as a transcriptional repressor for cellulase gene expression by binding at the *cbh1* promoter region, antagonized Xyr1 from binding to the *cbh1* promoter	[100]
BglR	It positively regulates the expression of genes encoding BGL in *T. reesei*. Its activation causes repression of the CBH and EG genes	[101]
ClbR	In *A. aculeatus* it regulates cellulase-encoding genes induced by cellulose and cellobiose	[102]
Ace3	In *T. reesei*, its overexpression led to increase cellulase gene expression, while its removal causes a remarkable reduction in cellulase activity	[103]
CreA/Cre1	They regulates CCR (carbon catabolite repression) where, in the existence of simple sugars such as glucose, fructose, mannose or xylose, it suppresses the transcription of genes encoding enzymes involved in the metabolism of more complex polysaccharides	[104]
Crz1	Through the conduction of Ca^{2+}/Mn^{2+} mediated signalling, it affects the promoter region of xyr1 and cbh1, and induced cellulase production	[105], [135]
Ctf1	In *T. reesei*, it downregulate RCE1 gene, thus it upregulate genes of the transcription factors XYR1 and ACE3, resulting in the activation of cellulolytic genes	[106]
Clr-1/Clr-2/Clr-3	Activation of the Clr-1 requires the presence of an inducer such as cellobiose, prompts expressions of bgl, but Clr-2 does not require any inducer for its activation. Abolition of them in *N. crassa* may prevent cellulose utilization as a carbon source	[107]
Clr-3	Clr-3 prevent the activity of Clr-1, removal of it led to the Clr-1 mediated gene expression in the absence of inducer	[108]
HAP complexes	HAP complexes are recognized in *Aspergillus* spp. (HapB/C/E), *T. reesei* (Hap2/3/5), and *N. crassa* (Hap2/3/5). They allow moderate expression of cellulase and xylanase genes in *T. reesei* (*cbh2* and *xyn2*)	[109]
Lae1 and VELVET complex	In *T. reesei* (LaeA orthologue in *A. nidulans*), it act as a methyltransferase induces cellulase expression and forming the VELVET complex, which in turn regulates the transcription of major cellulase genes	[110]
PacC/Pac1	In *A. nidulans* and *T. reesei*, it respond to pH variance in the external environment, stimulate or prevent cellulase production. At neutral pH, the abolition of the pac1 gene increases Xyr1 activity	[5, 55]
PoxMBF1	In *Penicillium oxalicum*, it binds directly to the promoter regions of principal cellulase and xylanase genes to induce cellulase production	[111]
PoxFlbC	In *Penicillium oxalicum* (FlbC orthologue in *Aspergillus*), it upregulates most of the cellulase genes	[112]
PoxAtf1	In filamentous fungi, it controls the expression of cellulase and xylanase genes during the solid-state fermentation	[113]
Blr1, Blr2, and Env1	Photoreceptor proteins (Blr1, Blr2, and Env1) in both light and dark regulate the cellulase gene from *T. reesei*. The photoreceptor regulative mechanism of carbon metabolism in *T. reesei* is dependent on Blr1 and Blr2 controlled by the Env1 photoreceptor	[114]
Seb1	In *Talaromyces pinophilus* EMU, Seb1 transcription factor binds to the stress response element (STRE) and CRISPR-Cas9 mediated degradation of *seb1* gene led to the 20–40% increment in FPase activity	[123]
TrAZF1	In *T. reesei* and *A. nidulans* positively regulates the activity of BGL and CBH by interacting with the *cel7a, cel45a*, and *sow* promoter region	[115]
PoSet1/PoSet2	In *Penicillium oxalicum*, PoSet1 and PoSet2 are responsible for methylation of histone (H3) lysine 4 and lysine 36, respectively. They are involved in the upregulation (PoSet1) or down regulation (PoSet2) of cellulase production	[116]

Table 2 (continued)

Transcription factors	Function	References
MAT1-2-1	It is a mating-type locus protein of *T. reesei* modulates cellulase gene expression in daylight by interacting with the Xyr1 and binds to the *cbh1* promoter	[117]

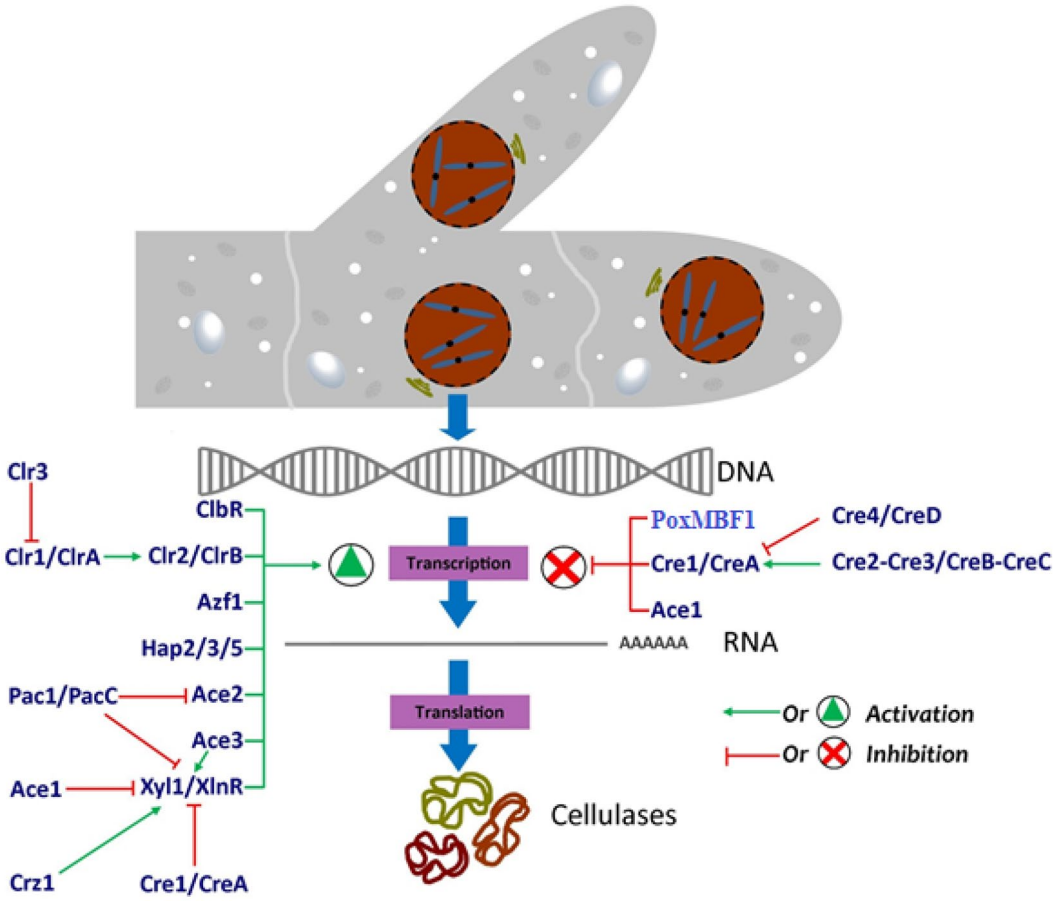


Fig. 1 Schematic overview of the transcriptional regulatory network of genes encoding cellulolytic enzymes in fungi

a fungal strain with an extraordinary saccharification efficiency and enzymatic activities for cellulose hydrolysis has remained a bottleneck. It is now renowned that a consortium of enzymes is obligatory for proficient degradation of cellulose; therefore, several genetic modifications of these fungi are needed for the cost-effective production of highly efficient cellulases, which has improved features such as higher yield, more excellent specific activity, superior stability (pH and temperature), and less susceptible to substrate-level inhibition [14, 23]. Several well-established genetic manipulation strategies (Fig. 2) in fungi for improved cellulase production are elaborated. A comparative account of different documented genetic manipulation strategies for betterment of fungal cellulase production was presented in Table 3.

Transcriptional regulators such as inducers and inhibitors profoundly affect gene expression. By adding or deleting such regulators, the expression level of the subjected gene can be substantially improved. TFs modification is a proficient strategy to increase enzyme production in fungi. The Xyr1 positively regulates cellulase induction since its abolition removes all possible inducers based on cellulase induction, while overexpression boosts cellulase induction [23]. Twenty six percent overexpression *xyr1* gene of *Trichoderma harzianum* prompts 36, 37 and 66% increment in the β-glucosidase, xylanase and FPase activity, respectively. The increment in enzymatic activities facilitates a 25% rise in the rate of sugarcane bagasse saccharification in the initial 24 h [24]. Besides, the constitutive expression of the *xyr1* (V821F) mutant led to a decrease in glucose repression [25] in *T. reesei*; therefore, enhanced cellulase and xylanase synthesis achieved on sugarcane bagasse [26]. Cre1 inhibits both the basal and inducible expressions as a principal

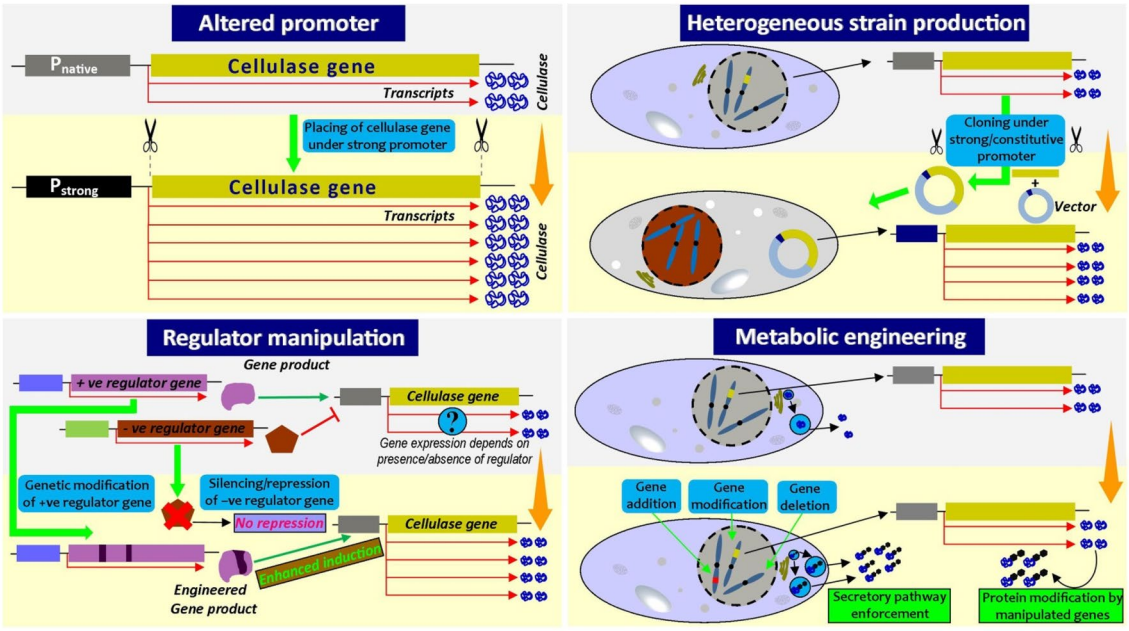


Fig. 2 Overview of different approaches of genetic and metabolic engineering for enhancement of cellulose production by fungi

negative regulator, thus maintaining the carbon catabolite repression (CCR) and avert *xyr1* gene expression. In *T. reesei*, the elimination of Cre1, which is responsible for suppressing carbon metabolism, is involved in the elevation of overall cellulase synthesis [27]. Zhang et al. [28] build a hybrid cellulase regulator involving the DNA-binding domain of the glucose-repressor Cre1 merged with Xyr1 effector binding domains, and overexpression of it led to the 30-fold increment in the constitutive expression of cellulase and hemicellulase when glucose was utilized as a primary carbon source in *T. reesei*.

RNA interference (RNAi) technology has been positively implemented in regulating the *cre1* gene expression in *T. koningii* led to the disturbing transcriptional level expression of *cre1* at varying degrees, resulting in increased activity of total cellulase level [29]. Besides, Wang et al. [30] also concomitantly executed RNA interference of Ace1 and overexpression of the positive regulator Xyr1 leading to many fold increments in extracellular protein secretion and filter paper activity in *T. reesei* Rut-C30 mutant. Moreover, artificial TFs have also been utilized for augmented cellulase production in *T. reesei*. Novel artificial TFs are synthesized by fusing several cellulases producing DNA-binding domains from positive and negative regulatory TFs in *T. reesei* with an activation domain of VP16 protein of herpes simplex virus. These TFs have incredible regulatory effects on cellulase production, improving the cellulase activity by 50%–80% than the parental strain in various inducing conditions [31]. Besides, novel minimal transcriptional activators having the DBD-Ace2 and the VP16 effecting domain outstandingly enhanced cellulase production, i.e., over 25-fold increment in FPase activity compared to the parental strain *T. reesei* Rut-C30, when utilizing glucose as the sole source of carbon [32]. Vib-1 has reported asserting regulation of cellulase synthesis in *N. crassa* [33] and overexpression of its homologous gene Trvib-1 in *T. reesei*, resulting in improved cellulase production [34].

Promoters play a pitotal role in the context of gene expression. Incorporating a strong promoter upstream of the gene of interest allows the desired level of gene expression. For manufacturing highly competent fungal strains of cellulase production, native promoters play a crucial role since cellulase expression is operated at the transcriptional level. Cellobiohydrolase 1 (CBH1) is the foremost secretory protein in *T. reesei*; therefore, the *cbh1* promoter has been widely used to induce hyperproduction of target proteins in *T. reesei*. It is known that the β-glucosidase (BGL) activity is deficient in *T. reesei*. It has been reported that in *T. reesei*, the application of such strong inducible promoters of *cbh1* and *cbh2* led to overexpression of BGL and other cellulases, which significantly expand the overall hydrolase activities [35]. Rahman et al. [140] reported that the activity of BGL has also been increased to many folds when the *bgl1* gene express from the *egl3* and *xyn1* promoters. The reorganization of cis-elements of Xyr1 in the *cbh1* promoter leads to a sevenfold increment in the *cbh1* promoter strength [36].

Similarly, in the manipulated *xyn3* promoter, the cis-acting area is exchanged to the cis-acting site of the *xyn1* promoter, improving the expression of *A. aculeatus bgl1*; therefore, amplified the overall cellulase hydrolysis efficiency

Table 3 Comparative analysis of different genetic manipulation strategies in fungi and enhancement in cellulase production

Strategy/target component	Modified fungal strain	Manipulation method	Outcome	References
Transcriptional regulators	*Trichoderma orientalis* EU7-22	Overexpression of the transcription factors Xyr1 and Ace3	2.12- and 1.95- fold increment in FPase (2.55 IU/mL) and CMCase (90.38 IU/mL)	[98]
RNA interference strategy	*Myceliophthora thermophile* ATCC42464	RNA interference of the *cre1* gene expression	3.76- and 1.31-fold increment in FPase and endoglucanase activity, respectively	[62]
Promoter engineering	*Aspergillus niger*	In *T. reesei*, the activation region of a strong inducible promoter P*cbhI* fused to a strongest promoter P*cdna 1–3* resulting in the production of a hybrid synthetic promoter P*cc*, followed by its heterologous expression in *A. niger*	43.2- and 1.2- fold increment in β-glucosidase (27.2 IU/L) and endoglucanase (6.2 IU/L) activity	[118]
Heterologous strain production	*Penicillium verruculosum* B1-537	β-glucosidase gene from A. niger and LPMO gene from T. reesei, clone in *Penicillium verruculosum* B1-537 and expressed under the inducible promoter gene *gla1*	β-glucosidase (11.8 U/mg) and LPMO (8.2 U/mg)	[41]
Codon optimization	*Trichoderma reesei* TU-6	Codon-optimized endomannanase gene (*Man5A*) transformation from *A. niger* to *T. reesei*	FPase (1376 IU/L)	[119]
Directed evolution	*Saccharomyces cerevisiae*	Employing directed evolution strategy to *bglI* gene from *A. niger* to express a library of mutated *S. cerevisiae bgl1* genes and utilized a two-round functional screen to recognize improved enzymes	(Variant7[a], mutational amino acid position 65, from Thy to Ade) 2.03 U/L (relative activity)	[52]
Multiple gene expression	*Aspergillus oryzae*	Multiple expression of *cbhI, egI*, and *bglI* genes	Cellobiohydrolase (21.8 U/L), beta glucosidase (37.7 IU/L), and endoglucanase (400 IU/L)	[53]
Signal peptide	*Pichia pastoris*	Replace the native signal peptide with signal peptide of serum albumin from *Homo sapiens*	Endoglucanse (261.45 U/mL)	[120]
Site directed mutagenesis	*Trichderma reesei*	SDM mediated disulfide bonds removal from *cel5A* gene	Endoglucanase (3111.7 U/mg for C99V and 3178.3 U/mg for C323H)	[121]
Chaperone	*Pichia pastoris*	Overexpression of the key transcription factor HAC1 that controls the unfolded protein response	Endoglucanase (91 IU/L)	[54]
Vesicle trafficking	*Saccharomyces cerevisiae*	Overexpression of vesicle trafficking components involved in ER to Golgi transport (Snc2p, Sec4p, and Ypt32p) and Golgi to plasma membrane (Sso1p and Snc2p)	20, 22 and 23% increment in endoglucanase activity and 53 and 61% in β-glucosidase activity	[122]
Glycosylation	*Trichoderma reesei*	Glycosylation at the N224 of β-glucosidase of *Aspergillus terreus* expressed in *T. reeesei*	β-glucosidase (450 IU/L)	[154]
CRISPR-CAS9	*Talaromyces pinophilus* EMU	CRISPR-Cas9 facilitated degradation of *seb1* gene	FPase (10.61 IU/mL)	[123]

[37]. The binding sites of transcription regulators are also manipulated to raise the production. Zou et al. [149] incorporated ACE2 and HAP3/HAP2/HAP5 by replacing the *cre1* binding site for the transcriptional activators inside the *cbh1* promoter, which led to a sevenfold improvement in cellulase expression. According to the report, the *cbh1* promoter is regulated by the carbon catabolite repressor (*cre1*), particularly when glucose is available. Promoter competence could be enhanced under the umbrella of CCR if the *cre1* binding sites can be substituted by the sequence where transcriptional activators bind. Wang et al. [38] established a promoter pool for the transformation of alkaline cellulase genes in *T. reesei* involved in multienzymes biosynthesis, which act together to alter the fabric surface as bio-stoning agent.

Heterologous strain production involves gene expression (or parts of it) of interest in a host organism lacking this gene or gene fragment naturally. A heterologous gene construct has been prepared by fusing *A. cellulolyticus* endoglucanase catalytic core with the catalytic core and linker of *T. reesei cbh1* and expressed in *T. reesei*. This recombinant protein product becomes functionally more efficient in cellulose hydrolysis of up to 20% within 6 h [150]. The Cel7A exoglucanase of *T. reesei* has been incorporated and expressed in *A. niger*. The recombinant strain is grown on spent hydrolysates (stillage) from sugarcane bagasse and can ingest Picea hydrolysate with enhanced endoglucanases activity than *T. reesei*. The cellulase gene cloned in *A. niger* was under a constitutive promoter to relieve the glucose repression in *T. reesei* strains [39]. For cellulase production in *T. reesei*, utilization of the repressor sugars present in molasses (sucrose, fructose, glucose), Ellilä et al. [26] has been developed a recombinant strain (VTT-BR-C0019) by expressing an improved Xyr1 TF under the constitutive pyruvate decarboxylase (*pdc1*) promoter. The manipulated strain exhibits increased cellulase production with the expression of an EG under the presence of the repression sugar glucose. In achieving higher level expression of the BGL, they cloned and overexpressed the Cel3A β-glucosidase from the moderate thermophilic fungus *Talaromyces emersonii* in the VTT-BR-C0019 to construct VTT-BR-C0020 strain. Furthermore, they cloned the invertase-expressing gene (VTT-BR-C0022) from *A. niger* to utilize sucrose in molasses. The final manipulated strain (VTT-BR-C0022) has shown better hydrolytic productivity utilizing lignocellulosic biomass with a concurrent drop in processing cost. A thermostable β-glucosidase from *Thermoascus aurantiacus* has been cloned and expressed in *Pichia pastoris*. The resultant recombinant yeast strain can be utilized cellobiose as a carbon source [40]. Simultaneous expression of *bgl1* encoding BGL from *A. niger* (AnBGL) and *egl4* encoding LPMO (previously endoglucanase IV) from *T. reesei* (TrLPMO) have been cloned in *Penicillium verruculosum* utilizing the inducible *gla1* promoter, led to the far better hydrolysis of lignocellulosic biomass in contrast with the wild type strain [41]. The *gpdA* promoter (isolated from *A. nidulans*) mediated overexpression of *clrB* in *P. oxalicum*, resulting in higher cellulase levels [42]. In the yeast strain *Pichia pastoris* X33, *bgl3* β-glucosidase gene from the *A. fumigatus* Z5 has been expressed using the vector pPICZaA, leading to a 3.5-fold increment in BGL activity [151]. Jäger et al. [131] have been reported to express *T. reesei* swollenin in *Kluyveromyces lactis*, and through microscopic observation, they found that swollenin regulates the breakdown of filter paper surface. Moreover, the crystallinity index of cellulose and filter paper has also been prevented by swollenin treatment. To improve the efficiency of a halostable cellulase isolated from marine *A. niger*, overexpression of EG and CBH genes was achieved through the promoter *glaA* and found CBH and EG activities improved from 0.21 and 4.51 to 0.89 and 15.12 U/mL, respectively [43]. A thermostable CBH gene from *Chaetomium thermophilum* has been cloned and expressed in *Pichia pastoris* led to the production of thermotolerant CBH, showing stability at 60 °C temperature and pH 5.0 [134]. Prabhu et al. [139] did similar work in *P. pastoris* and the resultant enzyme functions optimally at 60 °C in the pH range of 6.0–8.0.

Codon optimization is an approach for enhanced protein production based on two facts; the degeneracy of genetic code and species-specific codon usage bias of most amino acids. Numerous efforts for substituting the host expression system's favored codons with the rare codons have been fruitful to fortify the heterologous expression [44]. The heterologous expression of codon-optimized Cel6A of *T. reesei* was tenfold greater than the wild-type gene. Codon optimized *egl1* of *T. reesei* was expressed 1.24fold higher in *P. pastoris* [45]. *T. reesei* having the synthetic *cbh2* gene construct exhibits a 2.02-fold increment in cellobiohydrolase activity as compared to the native *cbh2* gene [46]. Phadtare et al. [47] constructed a recombinant *P. pastoris* by cloning and constitutively expressing a codon-optimized endoglucanase gene from *Myceliophthora thermophila* (Mt-*egl*) and attained sugar yields of 421 and 382 mg/g by hydrolyzing wheat bran and corn cobs, respectively. Pei et al. [48] executed site-directed mutagenesis to exchange the rare codons for the N-terminal amino acids of the *bgl* gene of *Thermoanaerobacterium thermosaccharolyticum* towards optimizing *bgl* codons for expression in *E. coli*. The recombinant BGL activity amended to 11.2 U/mg from 6.6 U/mg and showed higher-level tolerance to glucose and cellobiose.

Directed evolution is a strategy utilized to design proteins with necessary potentialities. This technique simply imitates natural evolution, involves selecting the target gene with subsequent random mutagenesis or molecular recombination, which led to the construction of a library of mutant genes. Further screening of the mutants is done for desired characteristics [152]. Improved catalytic efficiency,

thermostability, and glucose inhibition resistance of modified β-glucosidase (Ks5A7) was documented by Cao et al. [49] by substituting five amino acids in four cycles of random mutagenesis (through error-prone PCR) . One and half fold improvement of specific activity of the enzyme was noticed with high glucose tolerance (IC_{50} of 1.5 M). This mutant enzyme as a supplement with the Celluclast increased the glucose production by 44% following hydrolysis. Using the directed evolution strategy, Liu et al. [50] constructed a recombinant *E. coli* strain with a modified cellulase system, exhibiting an 8.2-fold rise in glucose yield compared to the cells armed with native enzymes. Here, the *cbh*A (exoglucanase gene from *Cellulomonas fimi*) mutant library fused with an expression vector (pET30a) carrying *bgl* gene (β-glucosidase from *T. reesei*) and transformed into *Escherichia coli* to form the entirely planned cellulase system. Using this strategy, Goedegebuur et al. [51] constructed a cellobiohydrolase I (Cel7A) from *Hypocrea jecorina*, which contained 18 mutated sites with a half-life 44 times better than the native form, and 10.4 °C rise of thermostability. A different version of β-glucosidase from the *A. niger* achieved through this tactic displayed Tyr → Cys substitution at 305 position. This point mutation led to a reduction in its transglycosylation activity, which otherwise inhibits its hydrolytic ability at high substrate concentrations [52].

Multiple gene expression is based on an evolutionary strategy, 'tandem gene duplication,' i.e., actually mimicking synthetic biology for enhanced protein expression. Recombinants obtained from genetically modified *Aspergillus oryzae* exhibit tenfold increment compared to the wild types (containing single gene) in cellulases activity. These were produced by integrating 5–10 copies of *cbh or egl*, or *bgl* genes into their chromosomes [53]. Li et al. [54] produced genetically engineered recombinants of straw mushroom with 262 and 151% higher endoglucanase activity harboring four and eight copies of the gene (*egl*) than the native hosts containing single and four copies, respectively.

The *signal peptide* sequence plays a crucial role in protein secretion. Substituting a potential signal peptide in a target protein tends to intensify its secretion efficiency [55]. Zhu et al. [56] increased EG activity (61.5%) by exchanging the innate secretory signal sequence of the endoglucanase I (*eg1*) gene by the *S. cerevisiae* MF*α* (α-mating pheromone). A gene cassette of β-glucosidase (*bgl1*) and endoglucanase II (*eglII*) respectively from *A. aculeatus* and *T. reesei* was developed by Inokuma et al. [57] for cell surface display. The signal peptide sequences of the cassettes were derived from *S. cerevisiae* SED1 (SED1SP), *Rhizopus oryzae* glucoamylase (GLUASP), and *S. cerevisiae* α-mating pheromone (MFα1SP). The modified strains with the SED1SP displayed 1.3- and 1.9-fold higher β-glucosidase activity than the GLUASP and MFα1SP strains, respectively. No significant change in extracellular endoglucanase activity of modified strains with the SED1SP and MFα1SP was noticed. The efficiency of engineered signal peptides is limited, not advantageous for all heterologous proteins [58]. Thus, identification of target protein-specific secretory fusion partner is mandatory. In yeast, for the poorly secreted proteins, an innovative protein secretion system was established based on the yeast genome-arrayed secretion leader library for searching the protein-specific translational fusion partners [58]. The degree of secretion and hydrolytic potential of *Chrysosporium lucknowense cbh2* were increased 2.4- and 1.4-fold than the native signal peptide (NSP) and MFα, respectively. The enzymatic activity of *bgl1* of *Saccharomycopsis fibuligera* was also 4.3- and 39.9-times higher than the protein secreted by the NSP and MFα, respectively [59].

Correct protein folding is one of the many prerequisites to protein secretion—molecular chaperon act to preserve nascent proteins in a folding-competent conformation and prevent aggregation. Therefore, *chaperones engineering* is an efficient tool for improving enzyme activity [60]. Tang et al. [61] observed a 53% rise in the specific activity of β-glucosidase of recombinant *Saccharomyces cerevisiae* strains than the wild type, resulting from overexpression of chaperone protein disulfide isomerase (Pdi1p) of endoplasmic reticulum. In a similar study Li et al. [54] documented increased folding and secretion of the recombinant endoglucanase in *Pichia pastoris* of up to 619% (91 U/mL), resulting from the overexpression of the key transcription factor HAC1 that controls the unfolded protein response. Yang et al. [62] cloned β-glucosidase (*bgl*) of *Thermoanaerobacterium aotearoense* together with a chaperone (groES-groEL) in *E. coli*. The recombinants exhibited a 9.2-fold better specific enzyme activity than the chaperone deficient strains.

Engineering of vesicle trafficking-related components is another strategy to facilitate heterologous protein expression, supporting the increment in cellulolytic efficiency. By overexpressing specific Soluble N-ethylmaleimide-sensitive factor Attachment Receptor (SNAREs) genes (responsible for encoding small membrane proteins that coordinate intracellular protein trafficking from Golgi to the cell membrane) in a recombinant strain of *S. cerevisiae*, resulting in enhanced secretion of its cellobiohydrolase (Cel7A) and β-glucosidase (Cel3A) isolated from *Talaromyces emersonii*, and *Saccharomycopsis fibuligera*, respectively [63]. The concurrent over-expression of the exocytic SNARE complex components having different combinations of Snc1/2, Sso1/2, and Sec9 genes led to the ~ 52% and ~ 49% increment in the secretion of Cel7A and Cel3A, respectively.

Trimming of *glycosylation* for improved cellulase yield is a new field of metabolic engineering [64]. As per rule, the N-glycosylation is a specific feature of catalytic domains (CD), whereas the peptide linkers, copious with serine

and threonine residues, are thoroughly ornamented with O-linked glycans [65]. N-linked glycosylation is the addition of glycan (an oligosaccharide) to amide nitrogen of asparagine residue of a protein. Glycosylation is also recognized to modulate binding to cellulose as well as lignin [66]. Adney et al. [67] observed 85% enhancement cellobiohydrolase-1 activity by adding an N-glycan on *Penicillium funiculosum Cel7A* at asparagine-194 (N194). Adney et al. [67] also reported that during expression of *T. reesei Cel7A* in *A. niger var. awamori*, removal of an N-glycosylation site (N384) resulted in a mutant strain with 70% higher activity than the *Cel7A* expressed in *T. reesei*. The deglycosylation of *T. reesei Cel7A*, resulting in the decline of enzyme stability [68]. Payne et al. [153] revealed that O-glycosylated cellulase linkers take part in cellulose-binding affinity, therefore, the O-glycans may be indispensable for cellulose binding [69] found that the addition of O-glycans resulted in improved thermostability, and cellulose-binding affinity of family 1 CBM. Wei et al. [154] studied the effect of four N-glycosylation sites (N224, NN295, N363, and N429) on the *Aspergillus terreus* β-glucosidase activity through heterologous expression in *P. pastoris* and *T. reesei*, and observed that the mutation on those sites of N-glycosylation led to reduced activity and thermostability.

CRISPR is chiefly a bacterial/archaeal immunity mechanism active against bacteriophage attacks. It is now applying as a powerful genome-editing technology [70]. The CRISPR system is broadly classified into two major classes: the class I system, which needs multiple effector proteins, while the class II system needs only single effector proteins. The class II system has either Cas9 or Cpf1 endonuclease [71]. The class II CRISPR system of *Streptococcus thermophilus* and *Streptococcus pyogenes* utilize a simple tactic using specific *Cas9* nucleases dependent on the species-oriented RNA-based adaptive immunity mechanism [72]. The single guide RNA (sgRNA), an amalgamation of crRNA (CRISPR-derived RNA) and tracer RNA (trans-activating CRISPR RNA), determines the target of Cas9. Cas9 identifies and cleaves the target DNA sequence based on sgRNA [16]. The sgRNA, which identifies a 20-nucleotide sequence located upstream of the protospacer adjacent motif (PAM), is fabricated as per the specific target of choice. Therefore, any preferred gene can be inserted or deleted through host repair mechanisms (i.e., homologous recombination and nonhomologous end-joining DNA), which quickly respond to Cas9 nucleases (Fig. 3). The Cas9 protein comprises of two domains: RuvC, which cleaves the DNA sequence non-complementary to the crRNA; whereas another domain, HNH cleaving the DNA strand complementary to the CRISPR RNA [73, 74]. Due to the marginal level of off-target effects, therefore, the Cas9 gene is extensively used in CRISPR technology [75]. The optimistic expression of Cas9 relies on the type of promoter. Hence, selecting an appropriate promoter is highly recommended for the CRISPR/Cas9 system [73]. In filamentous fungi, trpC, gpdA, and tef1 promoters derived from *A. nidulans* are often utilized [76, 77]. The Cas9 gene design is based on the bacterial or archaeal immune system, therefore, codon optimization is compulsory. Additionally, for utilizing this system in filamentous fungi, a specific signal sequence known as the nuclear localization sequence (NLS from SV40 is fused to either one end or both the end of Cas9 to import it to the nucleus through the nuclear translocation system [78]. Further, it plays a significant role in protein stability and checks outflow through nuclear pores [79]. A proficient CRISPR/Cas9 system is dependent on the expression of sgRNA. The sgRNA comprises no suitable CAP structure or poly (A) tail and cannot be adequately transcribed by the type II RNA polymerase. Due to a well-defined transcription site, the U6 promoters of type III RNA polymerase are often used in most filamentous fungi to express the sgRNA in the CRISPR/Cas9 system [80, 81]. However, there are some restrictions in utilizing the U6 promoters, for example, U6 snRNA are housekeeping genes ubiquitously expressed; thus, they cannot produce tissue-specific gRNA. Since RNA polymerase III is commercially not obtainable, U6 promoters are not appropriate for in vitro transcription of gRNA. Additionally, the CRISPR target sequences recognized by the U6 promoter have certain sequence specificity [$G(N)_{20}GG$] [82, 83]. Regarding this, both in vitro and in vivo approaches are applicable to express gRNA. One of the recently established approaches is constructing an efficient sgRNA expression system containing a 338-bp promoter sequence derived from the 5S rRNA gene of *A. niger* utilizing the sgRNA sequence as an expression cassette. An additional 88-bp sequence from *Trichoderma atroviride* was ligated between sgRNA and 5S rRNA to get off RNA interference [82, 84]. The CRISPR/Cas9 system having such a promoter for expressing sgRNA has been more efficient than U6 promoters [73] (Zheng et al. 2019). DSBs made by Cas9 can directly undergo indel mutagenesis by nonhomologous end-joining repair (NHEJ), or homology repair (HR) can be attained if a template for DNA repair is supplied [85, 86]. The poorly precise NHEJ repair occurs during the G1 phase of the cell cycle, while the exact HR repair pathway occurs only during the S phase. The ku70 and ku80 proteins mediated NHEJ repair pathways can be directly associated with the ends of the DSBs. The HR repair pathway requires a target site for homologous donor DNA fragments [87]. Additionally, the chance of HR repair will surge by around 1000 times if there are proximate homologous DNA fragments during DNA damage repair [73]. Therefore, an efficient CRISPR/Cas9 system can utilize the HR pathway for precise target editing, such as initiating specific point mutations at target sites, inserting a preferred sequence, or precisely substituting a sequence with the preferred sequence [88]. Liu et al. [89] constructed a CRISPR/

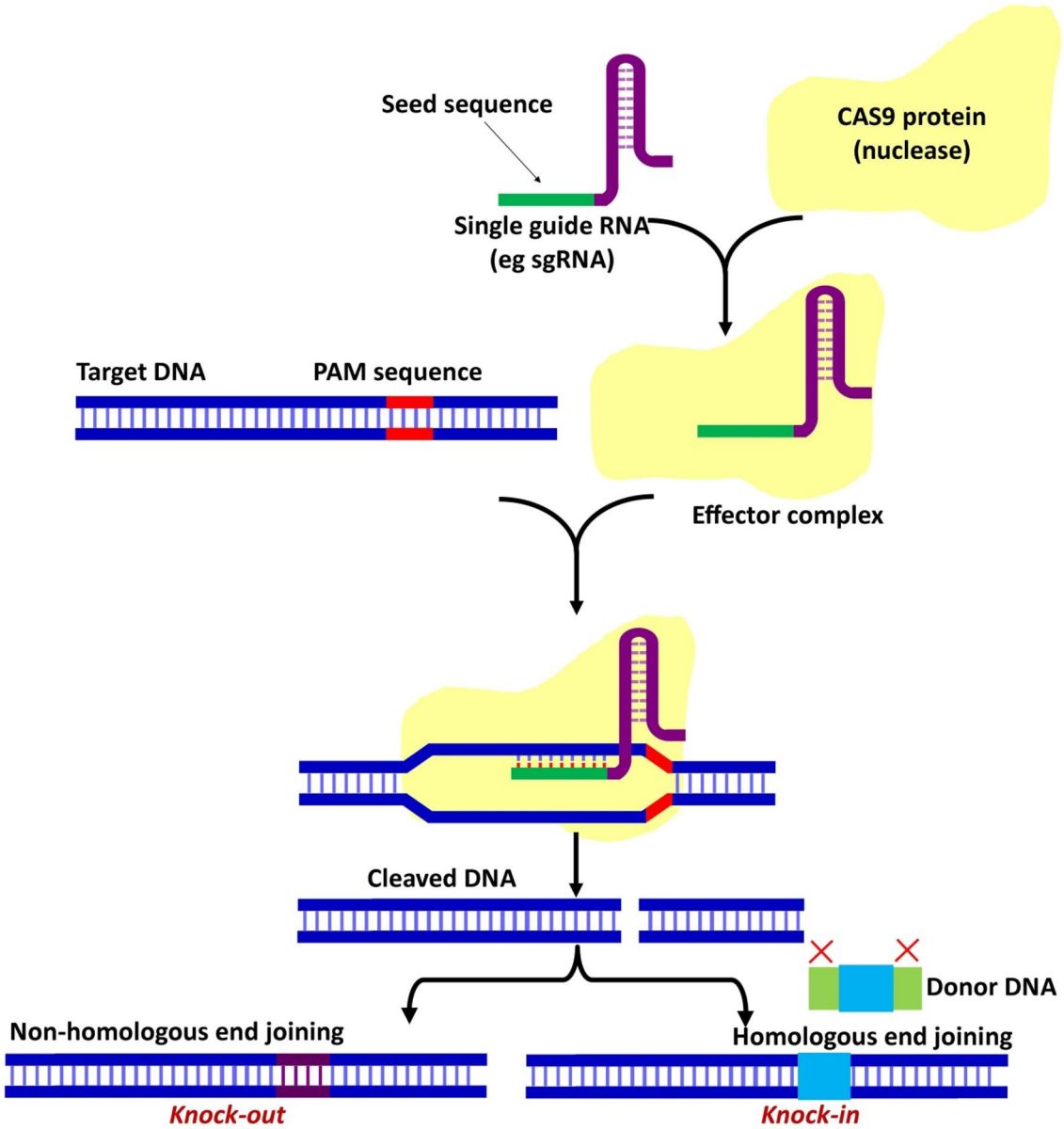


Fig. 3 Basic mechanism of CRISPR-CAS9 system

Cas9 system in *T. reesei* by introducing homologous arms of altered sizes to the left and right side of the selectable marker and revealed that the addition of this ≥ 600-bp homology arm around the selectable marker led to the 100% frequency of homologous recombination, facilitating CRISPR/Cas9-oriented gene knockout in *T. reesei*. Compared to the traditional genetic engineering strategies applied in filamentous fungi, the CRISPR-Cas9 system has several advantages. (a) It is simple in operation: a similar CAS9 can be applied to target different genes by altering the only sgRNA. (b) It is efficient: This system is applicable for quick manipulation of a single gene and used to amend multiple sites simultaneously. (c) It is flexible: it allows marker-free strain production, therefore assisting metabolic engineering of the strain by handling several genes. (d) It is non-toxic: No difference was observed in cell growth and sporulation when cells expressed Cas9 [90]. Matsu-Ura et al. [76] have been successfully executed CRISPR/Cas9 mediated genetic engineering of the filamentous fungus *N. crassa*, where the cas9 gene expressed under the control of the trpC promoter and terminator region from *A. nidulans* after incorporation into the genome of *N. crassa*. They utilize Cas9 endonuclease and single crRNA: tracrRNA chimeric guide RNA to place the *clr-2* gene under the control of a β-tubulin promoter, leading to approximately 200-fold increment in *clr-2* mRNA expression than the wild type strain, which subsequently

amplified the cellulase genes' expression. The *cbh-1, gh5-1* (*egl*), and *gh6-2* (*cbh*) mRNA expression levels augmented to 68.3, 1724.3 and 14.6 fold, respectively. Following this strategy, Liu et al. [90] engineered *Myceliophthora thermophila* and *M. heterothallica* strains having significantly greater potential of cellulase production. Multigene disruption of the cellulase production-related genes (*cre-1, res-1, gh1-1,* and *alp-1*) led to hypercellulase yield up to 5–13-fold. Abolition of *cre-1* gene from *M. thermophila* (1Mtcre-1) led to 3.7-fold additional protein secretion in avicel medium. Likewise, *M. heterothallica* mutant (1Mhcre-1) exhibited a noticeable enhancement in activity of cellulase and their degree of secretion. According to this technique, an engineered *S. cerevisiae* was created by incorporating 'sestc expression cassettes' having glyceraldehyde-3-phosphate-dehydrogenase gene promoter of *Agaricus bisporus* [91]. The endo-1,4-β-glucanase and exo-1,4-β-glucanase activities of the recombinants were 35.3 and 23-fold higher than the wild-type *S. cerevisiae*.

Cpf1 (previously Cas12a) denotes CRISPR-linked endonuclease derived from *Prevotella* and *Francisella*. Recently it gained more attention to the research fraternity as an emerging genome-editing tool that efficiently substitutes the CRISPR-Cas9 system [92]. It has several distinctive features compared to the CRISPR-CAS9 system. Cpf1 performed as a dual nuclease: as an endodeoxyribonuclease to cut target DNA and as endoribonuclease to process its own crRNA. It acts as a single RNA-guided endonuclease having only a short 42–44 nt long crRNA (lacking trans-encoded-crRNA) that identifies a thymidine-rich PAM at the 5′-side of the target site and introduces staggered cuts, resulting in a 5′-overhang distal to the PAM site. While, CAS9 endonuclease needs both 100 nt long crRNA and tracrRNA, recognizing guanine-rich PAM at the 3′-side of the target side and subsequently introducing blunt cut [92, 93]. Unlike Cas9 endonuclease, which has both the RuvC and HNH domains, the Cpf1 endonuclease comprises only the RuvC domain [92, 94]. Besides, Cpf1 permits multiplexed genome editing through its single crRNA array transcript that can target multiple loci in the genome [93]. For example, Abdulrachman et al. [92] showed that Cpf1 (FnCpf1) isolated from *Francisella tularensis*, introduced site-specific double-strand breaks at the pyrG gene in *A. aculeatus* TBRC 277, a potential enzyme synthesizing manufacturing hub. Thus, this strategy facilitates site-specific insertion or deletion followed by NHEJ.

To diversify the applicable potentiality of the CRISPR-CAS system, it is modified to a multiplexed CRISPR system that allows genome editing along with transcriptional regulation at multiple targets. Based on the catalytically inactive forms of Cas9 (dCas9) and Cpf1 (dCpf1), the traditional CRISPR-CAS system modified for activation of gene expression (CRISPRa) and gene interference (CRISPRi) [15, 95]. The catalytically inactive dCas9 have two point mutations in the RuvC and HNH nuclease domains, while the inactive dCfp1 has mutation only in the RuvC domain. Although such mutations inactivate the endonuclease activity of the CAS9 or Cpf1, they do not intervene in their normal binding to the guide RNA and subsequent interaction with the targets [15, 94]. By fusing to the appropriate transcriptional activators, the target-guiding skill of the CAS9 or Cpf1 endonucleases are utilized for gene activation, while the catalytically inactive enzymes after fusing to a repressor domain leading to transcriptional repression. Such approaches might benefit gene regulation in fungi, especially for multi-nucleated fungi and specific RNAi machinery deficient ascomycetes and basidiomycetes species [73]. Therefore, the CRISPRa and CRISPRi might be exploited for enhancing fungal metabolic pathways by concurrently activating or repressing genes that participated in targeted metabolic pathways [95].

Conclusion and prospects

Over the previous few decades, significant progress was observed in cellulase production, supporting a wide array of industrial purposes. Globally, cellulase apprises its market eligibility, drawing attention to developed and under-developed countries to implement a research strategy towards its commercial scale of production. Fungi being an inherent efficient producer and extracellular secretor of cellulases, is gaining intense research attention. Developing a fungal strain of improved cellulase activity with inexpensive production cost is the principal criterion of present cellulase research.

The varieties of biological systems' complexities may obstruct the pathway for designing novel approaches to support cellular potentialities for improving cellulase production. Advancement in systems biology and synthetic biology contributes to novel targets and ideas for the hyperproduction of cellulase by fungal strains. Competent genetic manipulation approaches have been developed from time to time to engineer filamentous fungi, and among these approaches, CRISPR/CAS system is most acceptable. At present, in fungi, the CRISPR/CAS technology has been providing varieties of applications: gene silencing, producing mutant, improvement of secondary metabolites, gene overexpression, gene regulation, gene tagging, and so on. However, there are still certain limitations in the applicability of this technology, such as the off-target effect, requirement in improving editing efficiency, and building an operational carrier system for several fungi. The progressive improvement in the CRISPR/Cas technology plays an indispensable role in the genetic modification of filamentous fungi in future research. Newish approaches of transcriptomics, fluxomics, metabolomics, and proteomics allow the innovative

concepts for the improved production of cellulase and make it an important tool for the sustainable development of modern human civilization.

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