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Minireview

Aspergillus spp., a versatile cell factory for enzymes and metabolites: Interventions through genome editing

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Aspergillus sp. is widely distributed in nature and plays significant roles in the degradation of lignocellulose biomass and extensively used in bioprocess and fermentation technology and many species are also a generally regarded safe. Many of the *Aspergillus* species are established cell factories due to their inherent capacity in secreting large number of hydrolytic enzymes. With the advent of next generation genomic technologies and metabolic engineering technologies, the production potential of *Aspergillus* cell factory has improved over the years. Various genome editing tools has been developed for *Aspergillus* like engineered nucleases, zinc finger nucleases, TALEN and CRISPR-Cas9 system. Currently, the CRISPR/Cas9-based technique is extensively used to enhance the effectiveness of gene manipulation in model system *Aspergillus nidulans* and other strains like *Aspergillus oryzae*, *Aspergillus niger* and *Aspergillus fumigatus*. This review describes the recent developments of genome editing technologies in *Aspergillus* the synthesis of heterologous proteins and secondary metabolites in the *Aspergillus* species.

Keywords: CRISPR, Genome editing

Introduction

Saprophytic filamentous fungi, especially *Aspergillus* genus, play a significant role in industrial biotechnology. This genus *Aspergillus* is consisting of over 300 species and have a significant influence on food synthesis, industrial biotechnology, and human health. The extensively studied genomics and metabolic functions of filamentous fungi make them extraordinary eukaryotic host for microbial cell factories[1,2]. The major advantage of a fungal production system involves its exceptional capability to synthesise and secrete a various variety of proteins and hydrolytic enzymes and its widespread use in fermentation technology[3,4]. They can cultivate on relatively cost-effective substrates like cellulosic biomass and also generate and secrete large number of enzymes and secondary metabolites.

The large number of available whole genomes sequence from several filamentous fungal strains including *Aspergillus* spp., has enhanced the possibility of genome modifications in filamentous fungi[5]. Recent developments in genome manipulation technology like, various selection markers, enhanced transformation efficacy, and enhanced gene deletion proficiency, amongst others[4], have significantly simplified the development of filamentous fungal productions hosts [5]. The advent of genome editing technologies like Zinc-finger nucleases (ZFNs) technology, transcription activator-like effector nucleases (TALENs) and clustered regularly interspersed short palindromic repeats (CRISPR) technology has revolutionised the area of filamentous fungal metabolic engineering for the production of eznymes and other secondary metabolites[6,7] .

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In this review, we have highlighted recent advances in basic and applied elements of on genome editing tools especially CRISPR/Cas, technique, its current trends, as well as future strain development programs in *Aspergillus*.

Genome editing in filamentous fungi and DNA double-stranded break repair in *Aspergillus*

Aspergilli are filamentous fungi that reproduce by asexual conidiospores and sexual ascospores[6]. *Aspergillus* fungi are distributed ubiquitously in different habitats of nature and are widely explored scientifically due to their importance in various fields. The advancements in molecular biology had made *Aspergillus* a potent substitute for eukaryotic hosts for the production of proteins of interest[7]. The industrially utilized strains of *Aspergilli* are improved beneficially with molecular tools so that the protein production can be controlled during transcription, post transcription, translation, and post-translation levels.

Several transformation techniques has been developed for *Aspergillus* like electroporation[8], biolistic transformation[9], and Agrobacterium-mediated transformation[10] were successfully done in *Aspergillus*. The introduced DNA will multiply by genome integration or replicate individually. Hygromycin, oligomycin, bleomycin, and phleomycin are commonly used antibiotic selection markers for *Aspergillus*[11]. Whereas nutritional selection markers include *acuD*[12], *amdS*[13], *prn*[14], *trpC*[15], *pkiA*[16], *pyrG*[17] and *argB* [18].

Genetic engineering deals with genome manipulation by inserting new genes, deleting/disrupting existing genes, and inserting or repairing point mutations. The selective genetic alterations are directly related to cell-based DNA repair. Various pathways are employed by eukaryotic cells to settle the DNA damage. DNA damage may be single-stranded breaks (SSBs) or double-stranded breaks (DSBs)[19]. DSBs being the lethal one, cells reverse this damage using NHEJ (Non homologous end joining), MMEJ (Microhomology mediated end joining), and HDR (Homology directed repair)[20]. These pathways are widely exploited for genome editing[21]. DNA is one of the complex macromolecules which is continuously exposed to harmful agents[22], and hence the genome stability strongly relies on the DNA repairing tools – NHEJ[23], MMEJ[24] and HDR[24]. NHEJ ties the ends of a DSB in a fallible way, with insertions and deletions. HDR copies the sequence from a repair template with flanking sequence homology for error-free DSB repair. In comparison, MMEJ makes use of short flanking regions (5-25 bp) of microhomology to repair DSB in DNA.

NHEJ and HDR are the most common type of tools for DNA repair in eukaryotes[25]. Ku heterodimer (Ku70 and Ku80), DNA dependent protein kinase catalytic subunit, and DNA ligase IV-Xrcc4 are the components of the complex which arbitrate the NHEJ process[26] resulting in random integration by the ligation of DNA strands sharing no homology[27]. The DSBs were identified by the Ku heterodimer which prevents further damage and signals to summon other units of the NHEJ pathway[28]. The strains of *Aspergillus* have enormous applications in industrial and clinical fields which are usually mutants lacking the Ku70 or Ku80 of NHEJ pathway. These fungal mutants are easily utilized for genetic modifications with better results due to the elevated frequency of homologous integrations. Genetic modifications by erasing NHEJ pathway components trigger the HDR pathway in mutants[29]. The deletion of human Ku heterodimer genes homologous in various *Aspergillus* species has been shown to activate the HDR pathway.

Targeted integration of genes is achieved in HDR by the interaction between homologous sequences aided by the RAP and Rad proteins [27]. In HDR 3′ overhangs are created by resection complex to which RPA is inducted which is further replaced by RAD51 supported by Rad52. Rad51 along with Rad55 and Rad57 helps in strand invasion. However, the HDR pathway is not much capable of doing DNA repair in *A. niger*[29]. MMEJ is also known as the 'alternative NHEJ' pathway and shares the conditions of the NHEJ and HDR pathways. MMEJ begins with resection and anneals the exposed microhomologies by deleting the intermediary sequence. MMEJ usually ends up with deletions, and sometimes results in translocations and insertions[30]. The deletions occurring in MMEJ are usually less protractile, as the MMEJ pathway uses only a short length of homology (5-25 bp) for repair [28].

Homologous recombination is an important and extensively adopted genomics tool for the production of gene knock-out mutants. However, the generation of homologous transformants in filamentous fungi like *A. niger* is tedious, as the frequency of homologous recombination is meagre in contrast to the *Saccharomyces cerevisiae* [31]. One hundred percent increase in homologous recombination frequency was reported in *Neurospora crassa*[26] by inactivating various

units of NHEJ pathway, and later the same strategy was well executed in other filamentous fungi[27]. MMEJ supports class switch recombination as it is independent of Ku and Lig D. The microhomology dependent MMEJ makes commodities that eliminate sequences among the microhomologies[20]. The list of *Aspergillus* species genetically modified by DNA repair mechanism is given in Table 1[12,28,31,34-73]

Engineered nucleases

Nucleases are enzymes that facilitate the cleavage of the phosphodiester bonds between nucleotides in DNA and RNA and are named as deoxyribonucleases (DNases) and ribonucleases (RNases), respectively[74] Nucleic acids can degrade single-stranded nucleic acids, double-stranded nucleic acids, or both. Exonucleases attack the 3′or the 5′ends of nucleic acid but not both. The endonucleases cleave the nucleic acid chain intermediately. Restriction enzymes are specific endonucleases that cut DNA at specific recognition sequences.

Table 1 — Genetic modification by DNA repair in *Aspergillus* strains

DNA repair tool	Mechanism	Organism with ref.
Non-homologous end joining	Deletion of Ku heterodimer (*Ku70*/*Ku80*)	*A. nidulans*[41,42]
		A. fumigatus[43,44]
		A. sojae[45-48]
		A. oryzae[49-50]
		A. niger[49,50]
		A. parasiticus[51]
		A. flavus[51]
		A. chevalieri var. *intermedius*[52]
		Neurospora strains[28]
		H. jecorina[53]
	Inactivation of *ligD*	*A. oryzae*[54-56]
		A. luchuensis[57]
		N. crassa[58]
Homologous recombination	*Agrobacterium tumefaciens*–mediated	*A. awamori*[12,59]
		A. fumigatus[60]
		A. giganteus[61]
		A. carbonarius[62]
	loxP site	*A. oryzae*[56]
		A. nidulans[69]
		A. fumigatus[70]
	Cas9	*A. niger*[34,38,39,71]
		A. aculeatus[34]
		A. brasiliensis[34]
		A. carbonarius[34]
		A. luchuensis[34]
		A. tubingensis[34]
		A. fumigatus[37]
		A. carbonarius[62]
Microhomology-mediated end joining	*glaA* deletion	*A. niger*[72]
	CRISPR mutagenesis	*A. fumigatus*[36,40]
		A. niger[73]
		A. oryzae[35]

The capability to alter the gene and protein performance is one of the key weapons used by molecular biologists to manipulate DNA for genome editing. This solely depends on the specificity of engineered nucleases that cleave precise genomic sequences in the target. Nucleases are the most successful reagents used in genome editing that specifically make DSBs in the target site[75]. The main nucleases used for genome editing are - Zinc Finger Nucleases (ZFN), Transcription Activator-like Effector Nucleases (TALEN), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) nucleases, and Mega Nucleases (MN)[76,77]. These nucleases make DSBs in specific gene sequence and the cell will repair these DSBs by NHEJ or HDR pathways.

Zinc finger nucleases

ZFNs contains a DNA-binding domain, derivative of zinc-finger proteins (transcription factors), and coupled to the nuclease domain of *Fok*I, a Type IIS restriction enzyme[78]. Each zinc finger (up to 6 fingers) of the DNA-binding domain is intended to identify and bind three nucleotides in the DNA sequence of the gene of interest. Similar to the parent nuclease, ZFNs have to dimerize to attach in DNA and cut it amid the binding sites[79], triggering the DNA repair tools – NHEJ or HDR. Natural *Fok*I, secluded from *Flavobacterium okeanokoites* having DNA-binding domain at N terminal and DNA cleavage domain at C terminal, identifies the 5′-GGATG-3′ sequence and cut delinquently the sense and antisense strand respectively later 9 nucleotides downstream and 13 nucleotides upstream of the recognition site[80]. However, when linked with zinc fingers the cleavage domain can be guided to a promptly preferred DNA sequence of our interest which will be different from that of *Fok*I[81]. Researchers have developed various combinations of ZFNs to identify a large extent of target DNA sequences[82]. Monomers of ZFNs have to dimerize to become active[83]. The linker length between the nuclease domain and zinc finger and the spacer length among the binding sites are important in the formation of the dimer[84]. The higher time and energy consumption for developing ZFNs and its limited target specificity restricts the usage of ZFNs in genome editing. The use of ZFNs in *Aspergillus* is summarized in Table 2[62,85-92].

Meganucleases

Meganucleases, also noted as homing endonucleases, usually identify 12 to 40 base pairs in the recognition

Table 2 — Engineered nucleases used for genome editing in *Aspergillus*

Nucleases	*Aspergillus* strain with refs.
Zinc Finger Nucleases	*A. nidulans*[85-89]
Meganucleases	-(no reports on *Aspergillus*)
TALEN	*A. oryzae*[90]
CRISPR/Cas9	*A. niger*[98]
	A. fumigates[92]
	A. carbonarius[62]

site of the target DNA sequence[93] and are therefore the most precise restriction enzymes existing naturally[94]. Meganucleases are found in phages, bacteria, archaebacteria and eukaryotes, and the same can be used to alter bacterial, fungal, animal or plant genome[93]. Even if meganuclease owns high level accuracy and a little toxicity, its target spectrum is narrow. Besides, the designing of meganucleases for interesting targets is difficult as the DNA-binding domain and nuclease domain is disheveled[95]. Depending on sequence and structure motifs meganucleases are classified into five families. The target specificity of meganucleases has been tried to increase by mutating definite residues.

Various research groups have used I-CreI and I-SceI as a platform to create mutants with different DNA recognition specificity[96]. The meganucleases used in genome editing create DSBs in the target DNA sequence and further activates the HDR DNA repair pathway[97]. I-*Ani*I is a homing endonuclease from *A. nidulans* used for genome editing[98]. Engineered meganucleases have been used to introduce homologous recombination yeast. The meganuclease genome editing[97] was established well from the experimental data obtained from budding yeast . However, to the best of our knowledge, we could not find any reports stating the use of meganucleases in *Aspergillus*.

Transcription activator-like effector nucleases

TALENs are structurally similar to that of ZFNs; with only disparity in the DNA binding domain which arrives from transcription activator-like effector (TALE) proteins from *Xanthomonas*, a plant pathogen[99] The DNA-binding domain is a squad of subdomains with amino acid repeats of approximately 34 amino acids with each recognizing a single base pair. The specificity of TALE is driven by the 12th to 13th amino acids that are hypervariable and are known as the repeat variable dinucleotide – RVD[100]. These RVD decides the binding to the probable nucleotides, denoting that one TALE sticks to single base pair only. The RVDs denoted as HD, NG, NI, and NN match up with C, T, A, and G, respectively (Joung and Sander, 2013). The TALE domain can be fused with various nuclease domains a variety of proteins including the *Fok*I[101]. TALENs dimerize to bind the target DNA sequence and create a cut in DNA, resulting in mutations.

TALENs are known to generate heterogenous overhangs which elicit an increased rate of deletions in the target sequence[90]. TALENs are sometimes chosen over ZFNs due to ease in delivery, improved binding to the site of interest, and suppler than triplet confined zinc finger proteins. On the other hand, the TALE cloning with desired sequences is very much challenging. TALENs with nonRVD variations (4th and 32nd residues) have better activity than conservative TALENs and these known as Platinum TALENs[102]. TALEN induced double-stranded breaks were made in yeast to make the mutants[103]. However, there are not many reports on the TALEN induce double-stranded break in *Aspergillus*.

CRISPR nucleases

The latest progress in the area of genome editing is the recognition of clustered regularly interspaced short palindromic repeats (CRISPR) along with the CRISPR associated (Cas) protein and is yet faster as well as commutable than the ZFN, TALEN and meganucleases[104]. Hence, the same has been described extensively in the following sections.

CRISPR/Cas9-mediated genome editing

CRISPR-Cas9 is the current and extensively used method for genome modification[91]. This is part of a defense system seen in bacteria and archaea[105]. These small DNA repeats separated by spacer DNA were first identified by Ishino *et al*.[106], in *E. coli*. Later in 2005, various researchers identified that these small DNA repeats are part of the immune system as the spacer DNA is of plasmid or viral origin[107]. The CRISPR–Cas systems arbitrate protection against breaching genetic components through the following steps — adaptation, expression and interference. Small DNA fragments, homologous to plasmid/virus, were incorporated into the CRISPR site in the adaptation step. In the expression step long primary transcript of CRISPR site (pre-crRNA) is produced and refined into short crRNAs, and in the final stage the targeted alien genome particle is destroyed[108]. CRISPR-associated proteins (Cas) usually possess nuclease, RNA binding, polymerase and helicase domains and are ciphered by presumed operons next to CRISPR sequences[109]. This makes them essential techniques for genome manipulation. Depending on

the involvement of Cas proteins, the CRISPR/Cas machinery was subdivided into Type I, II, and III[109].

The simple and broadly used type II CRISPR system[110] made up of a Cas9 nuclease, a target-recognizing CRISPR RNA (crRNA) and a non-coding trans-activating CRISPR RNA (tracrRNA) and RNaseIII[111]~~94~~. Later, crRNA and tracrRNA were connected together to make single guide RNA (sgRNA)[76] (Fig. 1). The Cas9-sgRNA complex creates a break (DSB) in the intended DNA constituting a 20 bp sequence corresponding to the protospacer of the sgRNA and a downstream protospacer adjacent motif (PAM) sequence[112]. Typically, PAMs are simply a stretch of a few nucleotides and vary among variants of the CRISPR/Cas system[113]. The sgRNA navigates Cas9 protein (having two DNA binding domains HNH and RuvC) to bind and cut the target sequence. The HNH domain cut the DNA strand dependent on crRNA, whereas the cleavage caused by the RuvC domain is independent of crRNA[114]. Thus, sgRNA identifies the 20 bp sequence upstream of PAM (at the 3′-end), and Cas9 create blunt end breaks in the DNA 4 bp upstream of PAM[91]. Then the genomic DNA instigates ascetic restoration via NHEJ or HDR pathway which has discussed earlier.

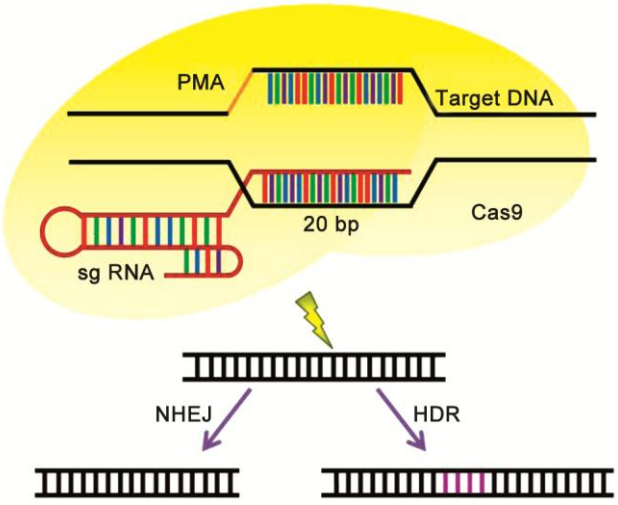

Fig. 1 — Schematic description of CRISPR/Cas9 system used for genome editing. [CRISPR/Cas9 system is made up of a Cas9 nuclease, a target recognizing CRISPR RNA and a non-coding trans activating CRISPR RNA. The Cas9-sgRNA complex generates double strand break in the intended DNA constituting a 20 bp sequence corresponding to the protospacer of the sgRNA and a downstream protospacer adjacent motif (PAM) sequence. The CRISPR/Cas9 system activates either NHEJ or HDR pathway to repair the break in the DNA]

Various species have been subjected to genome editing extensively by using CRISPR/Cas9 [115], with numerous applications in various fields[116]. CRISPR/Cas9 machinery helps to explore new levels of fungal research including filamentous fungi[117]. The technique was first initiated in *Saccharomyces cerevisiae*[118] and later on *Trichoderma reesei*[112], *N. crassa*[119] and *A. nidulans*[34]. Thereafter, the CRISPR/Cas9 genome editing technique has been used for altering the filamentous fungi genome, especially *Aspergillus*.

For fungal genome editing, the Cas9 codon is modified, a NLS signal is joined at 5′ and 3′ eds of the the Cas9 gene, and linked with sgRNA[34,112,120,121]. Usually, the Cas9 expression in the fungus is confirmed by the co-expression of a fused green fluorescent protein with Cas9[122-125]. Cas9 gene is usually transcribed by powerful constitutive promoters (trpC, gpdA, TEF1, xlnA, Ham34, amyB, niiA, Otef)[34-36,126-129]. However, for better controllability in the fungal system Cas9 is also transcribed under inducible promoters, such as Pcbh1 and PniiA[112,127]. Optimization of sgRNA is also important in fungal genome editing[130]. Functional sgRNA can be transcribed *in vivo* under the promoters of RNA polymerase II and III[119,131]. For *in vitro* transcription of functional sgRNA, U6 and T7 promoters of RNA polymerase III are used and finally form the ribonucleoproteins to cute the DNA[36].

For the fungal genome editing based on CRISPR/Cas system, Cas9 and sgRNA expression vectors must be incorporated in the fungal cells. The vectors can be delivered either as a single vector carrying both Cas9 and sgRNA or as individual vectors with each expression cassette of Cas9 and sgRNA. The efficacy of both the single and the dual vector system has been confirmed in *A. fumigates* and the results showed that the single-vector expression system is better in precision and effectiveness[36]. Hence, single vector system is preferred in fungal genome editing where fungal cells were initially transfected with vectors carrying Cas9 expression cassettes and further Cas9 positive cells were transfected with sgRNA expression cassettes[38,39,112,123]. Usually, the fifty percent of the CRISPR/Cas9 induced mutations are solitary insertions and the remaining are minor deletions up to 50 bp[131,132]. The CRISPR/Cas9 machinery induced gene knockouts are predominantly single-gene insertions or small fragment deletions in case of

filamentous fungi[133]. The main drawback of the CRISPR/Cas9 system is its off-target specificity. PEG mediated transfer of stable Cas9 and sgRNA complex reduces off-target specificity in filamentous fungi[134]. Sequencing techniques such as ChIP, Digenome, and GUIDE can be utilized to detect the off target points[111,135,136]. Different *Aspergillus* strains engineered by CRISPR/Cas9 are as follows: *A. niger*[38,39,137], *A. oryzae*[35], *A. fumigatus*[40], *A. aculeatus*[34], *A. brasiliensis*[34], *A. carbonarius*[34], *A. luchuensis*[34], *A. nidulans*[138], *A. luchuensis*[139] and *A. carbonarius*[62].

Implications of genome editing in *Aspergillus* cell factory development

The advent of CRISPR/Cas9 technology has revolutionised the field of filamentous fungal genome engineering of various *Aspergillus* spp.[140]. CRISPR/Cas9 technology offer accurate gene editing and engineer fungal hosts for desired traits. Modified versions of CRISPR/Cas9 enzymes can also be used for many applications in epigenetic modification and DNA nicking[141]. CRISPR has the capability for extensive application in examining the expression of fungal genes, especially for the genes responsible for the biosynthesis of secondary metabolites. Many of the *Aspergillus* species are efficient producers of bioactive natural compounds. Many of the bioactive synthesis genes are clustered in a particular locus and not expressed under normal cultivation conditions[142]. Nonetheless, bioactive metabolites synthesised by a variety of cryptic clusters of metabolite genes also be elucidated. The advancement of CRISPR/Cas9 technologies might help as an efficient tool for identifying the compounds synthesised by the clusters of secondary metabolite gene. While many of the recently established CRISPR/Cas9 platform were applied mainly for the function-based characterisation of variety of filamentous fungal genes, and other documented applications are in the development of *Aspergillus* cell factory, bioenergy production and investigating gene regulation[143].

Implications of CRISPR/Cas9-assisted gene disruption

Initially advancements in CRISPR/CAS9 technology in filamentous fungi applied typically on creating gene disruption using non-homologous end joining (NHEJ) and homologous recombination (HR) strategies. The abundance of large number of target genes in filamentous fungi makes the rapid developments in CRISPR/Cas easy. Typically, this genome editing system involves the targeting of genes involved in the production of pigments or antibiotic resistance genes, because they have observable phenoptypes after gene disruption. The different pigment synthesis genes that was disrupted involve *A. nidulans yA* gene, *A. niger alba* gene[34], *A. fumigates pksP* gene and[40] *A. alternate pksA* gene[144]. These findings showed that the most of NHEJ's gene targeting involved either nucleotide deletions or insertions at the cleavage site of Cas9 which results in frame shift mutations. Disruption of coding sequence of target gene by HR involves the incorporation of a dominant selection marker which confers resistance to fungi in growth medium.

***A. niger* cell factory as a typical example**

A. niger is the world's largest producer of citric acid and is applied in various industries like food and pharmaceutical. Advancement in the field of *A. niger* genomics and proteomics greatly enhanced the understanding of citric secretion in *A. niger*. The introduction of CRISPR/Cas9 system in filamentous fungi enables extremely proficient genome-level gene manipulation in *A. niger*. Nowadays, numerous CRISPR/Cas9 genome manipulation techniques were introduced in *A. niger*. Recently Nodvig *et al*.[34] described the foremost CRIPSR/Cas9 genome editing in *A. niger*. They constructed a single plasmid with expression cassette for Cas9 and sgRNA with the help of RNA polymerase II promoter p*gpd*A. The developed system allows the NHEJ-mediated targeted gene disruption. Kuivanen *et al*.[38,39] developed a sgRNA expression cassette using T7 promoter and then sgRNA was co-transformed along with Cas9 expression construct into the *A. niger* protoplast. The newly constructed gene editing platform was superior to attain fast editing of fungal genome, but the effectiveness was inclined by the sgRNA stability and efficiency of transformation[127]. Zheng *et al*. (2018)[137] introduced U6 promoter for sgRNA and verified the efficiency in disruption of gene. All the verified U6 promoters allowed the transcription of sgRNA and subsequent gene disruption but with less transformants and low gene disruption efficiency. Later, they constructed anew CRIPSR/Cas9 system with promoter of 5S rRNA for sgRNA expression[145]. This resulted in the 100% gene disruption efficiency with homologous recombination. This newly developed system has been useful for design of chromosome, as established by the insertion of multiple genes and deletion of huge fragment of DNA to reduce the mycotoxin formation in *A. niger*.

Furthermore, CRISPR/Cas9 based editing of genome was applied along with transcriptome technique for the production of *A. niger* galactaric acid. Particularly, researchers identified six genes responsible for the catabolism of galactaric acid. Then they deleted all these genes by homologous recombination and found that galactaric acid production was blocked in these mutants. Then mutants with no galactaric catabolism was selected and engineered to enhance galactaric production[38]. Huang *et al*.[146] developed a series of single-base editing tools that convert cytidine to thymine without any double stranded in *A. niger*. This was done by combining cytidine deaminase and Cas9 nickase. They disrupted uridine *pyrG* auxotrophic gene (uridine) and pigment gene *fwn*A with high efficiency.

A. nidulans and *A. oryzae* cell factories for bioactive secondary metabolites

A. nidulans was tested as the heterologous host platform for evaluating the *A. fumigatus* EAS pathway. Ergot producing EAS pathway is absent in *A. nidulans*. Chanoclavine-I was heterologously expressed by the mutants of *A. nidulans* transformed with heterologous genes like *dmaW*, *easF*, *easE*, and *easC* from *A. fumigatus* EAS gene cluster and the expression of genes are with the help of native promoters. In order to examine the candidate genes in the pathway, several *A. nidulans* mutants were created by transforming the fungi with different combination of gene cluster like *easE*, *easC* and *dmaW*, *easF*. These mutations studies indicated the importance of *easE* and *easC* for the production of chanoclavine-I by the conversion of N-Me-DMAT[147].

Disruption of gene clusters responsible for secondary metabolite production decreases the chances of producing unwanted metabolites in the host strain. In a modified *A. nidulans* strain the gene clusters for secondary metabolites emericellamide, sterigmatocystin, asperfuranone, orsellinicacid, terrequinone and monodictyphenone were disrupted. *pyrG* from *A. fumigatus* was used as the selection marker. NR-PKS (Nonreducing polyketide synthase) secondary metabolite cluster genes from *A. terreus* were cloned in fragments combined with various selection markers, and transferred into the mutant of *A. nidulans* for HR mediated targeted gene integration and the entire NR-PKS gene cluster was produced. Asperfuranone (*afo*) biosynthetic pathway was introduced in *A. nidulans* from *A. terreus* were studied. The genes were combined with strong promoters which are regulatable. This resulted in the finding of order of genes in the asperfuranone biosynthetic gene cluster which is *foC*, *foD*, and *foF* their role in biosynthesis. Thus *A. nidulans* is a versatile host for the production of fungal secondary metabolite gene clusters[148]. *A. oryzae* is another widely used expression host for heterologous protein because of GRAS status and exceptional secretion machinery for secreting various hydrolysing enzymes. Several non-ribosomal peptides, polyketides, terpenoids were heterologously expressed in modified strains of *A. oryzae*[149]. Trypacidin biosynthetic gene cluster was constructed in *A. fumigatus* with help of CRISPR/Cas9 and computational techniques. In this study they have used doxycycline-inducible tetON system for Cas9 gene expression[92]. Recently, Roux *et al*.[150] established a CRISPR/dLbCas12a-VPR-based gene disruption system and established the expression of a fluorescent reporter in *A. nidulans*. Then, they directed the native NRPS gene (nonribosomal peptide) micA in the chromosome. This enhanced the production of the secondary metabolite, microperfuranone. Lastly, multi-gene CRISPRa resulted to the detection of the *mic* gene cluster product as dehydromicroperfuranone. They also investigated the different parameters that affect the efficiency of CRISPRa in fungi.

Conclusion

Various species of *Aspergillus* possess potential characteristics and thereby exploited commercially for the synthesis of various organic acids, enzymes and recombinant proteins. Various genome editing techniques have been established in filamentous fungi which enhances the metabolite and enzyme production. To further improve the production capability and to study physiological aspects of *Aspergillus* spp. efficient implementation of genome editing tools are necessary. Genome editing with the ZFN, TALEN, mega nuclease, and especially CRISPR is an emerging field consistently yielding productive results by manipulation of the *Aspergillus* genome. These state-of-art techniques have contributed significantly to the enhancement in the expression of target genes and pathway.

Conflicts of interest

Authors declare no competing interests.

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