Received: 19 March 2024 | Revised: 25 June 2024 | Accepted: 29 June 2024

DOI: 10.1002/biot.202400180

Biotechnology Journal

REVIEW

Synthetic biology for *Monascus*: From strain breeding to industrial production

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Funding information
National Key Research and Development Program of China, Grant/Award Number: 2022YFA0911800; Zhejiang Provincial Natural Science Foundation of China, Grant/Award Number: LQ22C010001

Abstract

Traditional Chinese food therapies often motivate the development of modern medicines, and learning from them will bring bright prospects. *Monascus*, a conventional Chinese fungus with centuries of use in the food industry, produces various metabolites, including natural pigments, lipid-lowering substances, and other bioactive ingredients. Recent *Monascus* studies focused on the metabolite biosynthesis mechanisms, strain modifications, and fermentation process optimizations, significantly advancing *Monascus* development on a lab scale. However, the advanced manufacture for *Monascus* is lacking, restricting its scale production. Here, the synthetic biology techniques and their challenges for engineering filamentous fungi were summarized, especially for *Monascus*. With further in-depth discussions of automatic solid-state fermentation manufacturing and prospects for combining synthetic biology and process intensification, the industrial scale production of *Monascus* will succeed with the help of *Monascus* improvement and intelligent fermentation control, promoting *Monascus* applications in food, cosmetic, agriculture, medicine, and environmental protection industries.

KEYWORDS
filamentous fungi, gene editing, *Monascus*, *Monascus* pigments, solid-state fermentation

1 | INTRODUCTION

Filamentous fungi are ubiquitous organisms with powerful abilities to synthesize diverse structural compounds with broad biological activities, which would help improve the social and environmental problems, enabling us to achieve carbon neutrality and a circular economy, with applications ranging from plastic degradation to animal protein substitutes. At the same time, secondary metabolites synthesized by filamentous fungi can be used as drugs, plant growth regulators, pigments, etc. Still, most of these compounds contain mycotoxins, posing risks to livestock and humans. The mycotoxin biodetoxification through degradation and adsorption by microorganisms or enzymes has attracted many studies, including the mycotoxin elimination at the source by the metabolic engineering of the fermentation strain.[1,2] In addition to addressing mycotoxins, many vital drugs achieved through downstream purification have been developed and applied in clinical treatments, including penicillin, statins, and antifungal medicines such as echinocandins. In recent years, the application scope of filamentous fungi has broadened, particularly in the green synthesis of gold nanoparticles (AuNps),[3] leveraging their unique secretion metabolites and proteins. *Trichoderma viride*, for instance, has proven to be an effective source for AuNps, yielding biocatalysts with antibacterial activities against pathogenic bacteria.[4] Other studies also show that filamentous fungi are important pillars for the green and sustainable developments of biotechnology.[5–7]

Red yeast rice, fermented rice with *Monascus*, has been used as traditional Chinese medicines, food colorants, and additives for more than 1000 years in China, making it a valuable natural and healthy

filamentous fungus.[8–10] *Monascus* is a small saprophytic filamentous fungus that is acid-resistant and ethanol-resistant, with its optimum growth pH at 3.5–6.0 and optimum growth temperature at 25–32°C. *Monascus* can produce valuable chemicals in the fermentation process, such as *Monascus* pigments (MPs, natural pigments used in food and cosmetics),[11] lovastatin (hypolipidemic drugs),[12,13] ergosterol (important chemical used in pharmaceutical and chemical industry),[14] dimerumic acid (DMA, antioxidant),[15] γ-aminobutyric acid (GABA, important inhibitory neurotransmitter), and so on, which shows the great potential for *Monascus* in pharmaceutical, food, nutrition, cosmetic, and other areas.

The number of fungal species undergoing genome-wide sequencing is rapidly increasing to enrich the knowledge resources of filamentous fungi, especially through projects such as the 1000 Fungal Genomes Project or the *Aspergillus* Whole-genus Sequencing Project.[16] However, due to the relatively large genome sizes, complex cellular structures of fungi, and difficulties in gene manipulation,[17] the development of filamentous fungi is seriously hindered, especially by the lack of genetic markers that makes their genetic engineering difficult,[18] as well as the low gene targeting frequencies that render reverse genetics tedious when functional markers are available, thereby posing major bottlenecks for fungal genetic characterization.[19] Therefore, it is necessary to establish and improve the genetic transformation system of filamentous fungi to provide convenient technical support for gene studies and manipulations. Gene editing technology has broad application prospects, including disease treatments, improving agricultural food supply, improving the production efficiency of scarce biological products, solving climate issues, and controlling the number of pests and diseases.[20–23] By studying the functions of genes related to the biosynthesis of valuable chemicals in filamentous fungi and applying biotechnology to genetic breeding or modification, the abilities of filamentous fungi to synthesize these chemicals can be significantly improved, thereby promoting their industrial applications. Gene knockout technologies based on homologous recombination (HR), such as Split marker and λ Red, have been widely used in the study of gene manipulations in filamentous fungi.[24,25] However, due to factors such as low efficiencies of HR, high mismatch rate, the need to screen many transformants, and colossal workload, the CRISPR tool has gradually replaced it. Recently, researchers have successfully developed various endonuclease technologies for gene editing, including zinc finger nuclease (ZFN), transcription activator-like effect nuclease (TALEN), CRISPR/Cas9, and other technologies.[26,27] Compared to ZFN and TALEN technologies, gene editing technology using CRISPR/Cas9 shows the advantages of simple operation, good targeting, and high safety, which helps it become the preferred technology for gene manipulations of filamentous fungi.[28,29]

This article summarizes the most commonly used techniques in the metabolic engineering of filamentous fungi, followed by an overview of the current industrial production strains of MP and lovastatin and the elimination of citrinin (CIT). We also discuss the key issues that occurred in the metabolic engineering of *Monascus* to provide a theoretical basis for applying synthetic biotechnology in the metabolism of filamentous fungi. With the prospect of *Monascus* scaling up fermentation technologies, researchers will quickly implement *Monascus* applications from laboratory to industry.

2 | DEVELOPMENT OF GENE EDITING TOOLS FOR FILAMENTOUS FUNGI

Filamentous fungi exhibit distinct physiological traits from bacteria, including apical growth, heterokaryosis, low HR rates, and a dearth of genetic markers, hindering the establishment of advanced genome editing platforms.[30,31] Owing to the scarcity of genetic markers in fungi, multi-genome editing tools rely on marker cycling techniques like Cre/*loxP* and FLP/*FRT* to sequentially edit target genes.[32–34] However, marker cycle techniques generally restrict the number of editable genes and take a long time. Hence, developing efficient gene editing technologies capable of targeting multiple genes across filamentous fungi, including *Monascus* species, is crucial for advancing their industrial applications. The development of synthetic biology for constructing engineered flamentous fungi would also help expand their industrial applications, which can be devided into six types: food and cosmetic additives,[35–38] ecological restoration,[39,40] biobased material,[41] microbial control,[42,43] biological conversion,[44] and nutraceuticals (Figure 1).[45–47]

2.1 | Cre/*loxP* tool

The Cre/*loxP* recombinase system facilitates precise site-specific DNA recombination, leveraging the Cre recombinase and *loxP* site from P1 phage. Cre recombinases belong to the integrase family of site-specific recombinases, responsible for recognizing the *loxP* site and catalyzing precise site-specific recombination of DNA between the two sites.[48] The recombination reaction is a reversible dynamic equilibrium process, where the direction of the two *loxP* sites determines whether the intermediate DNA sequence undergoes deletion, insertion, inversion translocation, or cassette exchange (Figure 2I).[49] The Cre/*loxP* recombinase system is the preferred conditional gene knockout tool, renowned for its simplicity. It operates without additional cofactors or energy expenditure, relying solely on recombinase and site recognition, contributing to its widespread use. Researchers can also regulate the recombinase expression in this system to realize the transformation of target genes in specific tissues or at a specific time.[50] Like the Cre/*loxP* system, the FLP/*FRT* system derived from the yeast *Saccharomyces cerevisiae* is composed of an FLP recombinase and corresponding *FRT* sites.[51] It can also be used for marker recycling in filamentous fungi.[34]

Among the advantages of using the Cre/*loxP* system, the chromosomal integrations of large biosynthetic gene clusters are attracted. The targeted integration of a 21 kb DNA fragment in *Aspergillus nidulans* based on Cre/*loxP*-mediated cassette exchange was successfully carried out,[52] indicating its potential application for extensive DNA fragment integration in *Monascus*. In addition, Cre recombinase has also been used in *Aspergillus oryzae*,[33] *Aspergillus niger*,[53]

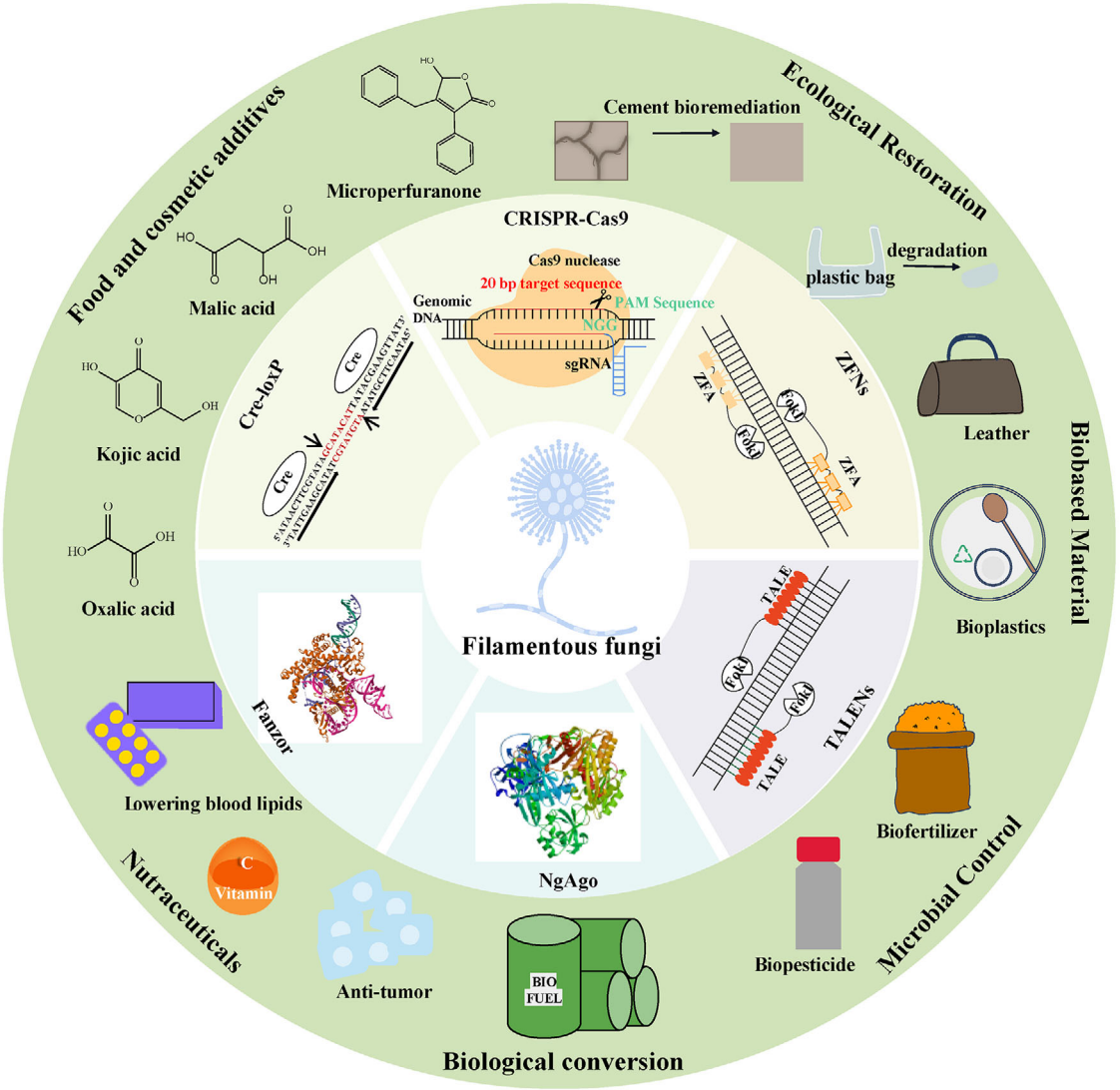

FIGURE 1 Partial gene editing tools and applications in filamentous fungi.

Trichoderma,[54] and so on. Although the gene editing efficiencies using Cre/*loxP* systems differ from the target genes in filamentous fungi, most of the genes can be edited with over 50% efficiency, as shown in Figure 2IV. However, the use of Cre/*loxP* and FLP/*FRT* systems results in residual *loxP* or *FRT* sites, which will reduce the genomic stabilities, especially for multiple gene manipulations. In summary, the Cre/*loxP* site-specific recombination system has made it possible to precisely design genetic modifications, allowing for the precise introduction of foreign genes and setting genetic switches to control the expression and deletion of foreign genes in time and space, demonstrating enormous application prospects.

2.2 | ZFNs and TALENs tool

ZFNs technology is the first generation of genome editing technology, composed of zinc finger proteins (ZFPs) with specific recognition sequences and FokI endonucleases. The ZFP-based DNA recognition domain recognizes and binds to specific DNA sites, while the FokI-composed cleavage domain executes splicing, enabling the targeted double-stranded DNA to break. And with the help of HR repair mechanisms and non-homologous end joining (NHEJ) repair mechanisms, cells can repair their DNA.[26] HR repair may involve restoration or insertion modifications to the target site, while NHEJ repair is prone to insertion or deletion mutations. Both can cause frameshift mutations, thus achieving the goal of gene knockout.

Transcription activator-like effector nucleases (TALENs) are another flexible and efficient genome editing technology after ZFNs. Like ZFNs, TALENs consist of zinc fingers as specific DNA-binding sites and Fok I nuclease. The TALE protein is a transcription activator-like effector secreted by the plant pathogen *Xanthomonas* that can recognize and bind to the sequence of host target genes in plant cells, thereby regulating host gene expressions.[55] During genome editing, two TALE proteins recognize and bind to DNA target sites. Fok I nuclease is responsible for cutting the target DNA, resulting in a DNA double-strand break (DSB) and inducing DNA damage repair

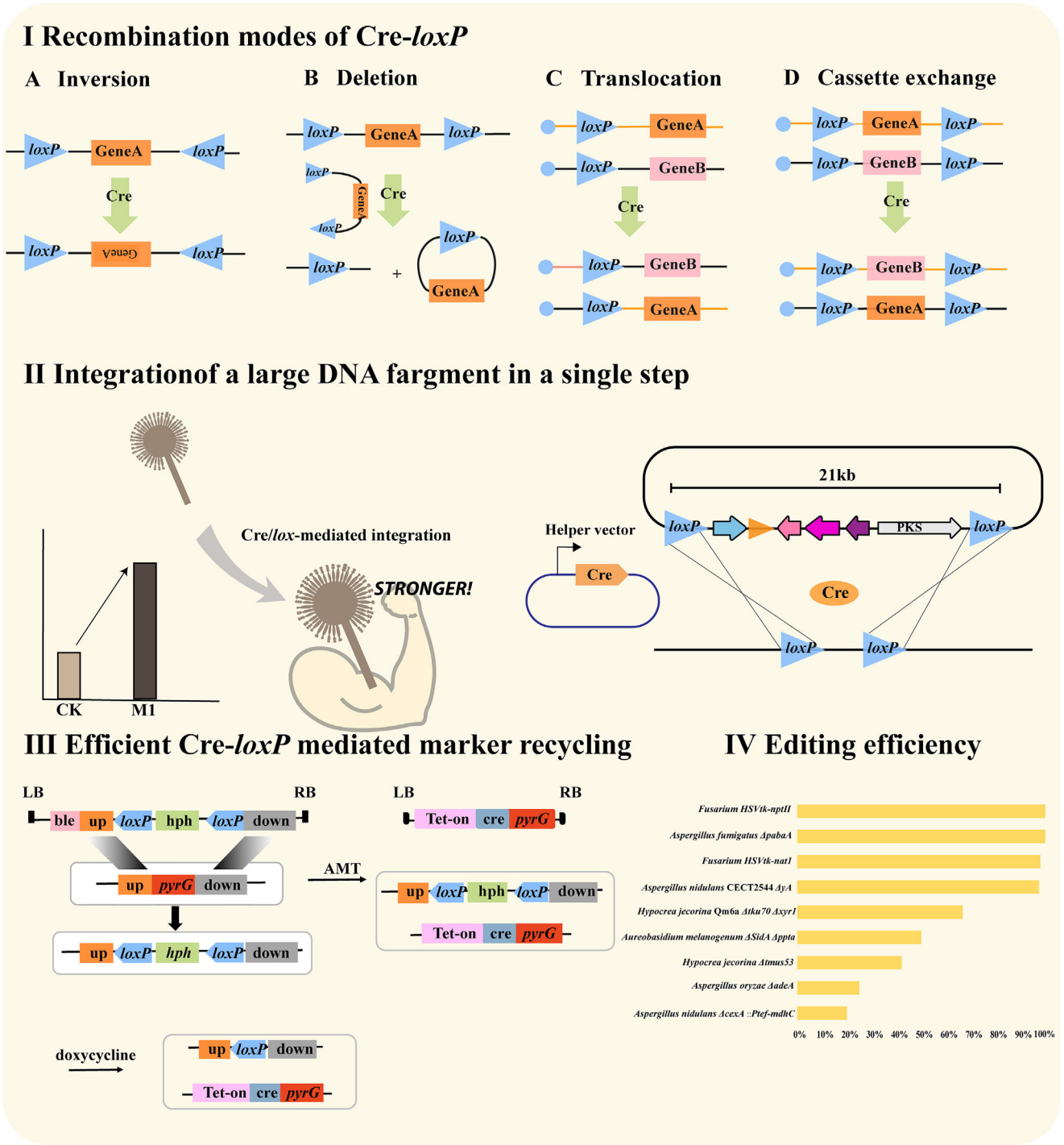

FIGURE 2 Principle and application of Cre-*loxP*. (I) Recombination modes of Cre-*loxP*. (A) Cre recombinase mediates sequence inversion between *loxP*s if the two *loxP* sites are located on the same DNA strand and in opposite directions. (B) Cre recombinase mediates sequence excision between *loxP*s if both *loxP* sites are located on the same DNA strand and in the same orientation. (C) Cre recombinase mediates the exchange of two DNA strands or chromosomal translocation if the two *loxP* sites are located on different DNA strands or chromosomes. (D) Cre recombinase mediates the cassette exchange if the four *loxP* sites are located on two different DNA strands or chromosomes. (II) Integration of a large DNA fragment in a single step.[52] The landing pad containing *loxP*s is first integrated into the targeted locus of the *Aspergillus nidulans* genome by homologous recombination, followed by transformation of the prepared protoplasts with the donor vector. (III) Efficient Cre-*loxP* mediated marker recycling.[193] AMT, agrobacterium-mediated transformation; *ble* and *hph*, bleomycin and hygromycin; Doxycycline, the chemical used to induce the expression of cre in stains; *pyrG*, orotidine 5′-phosphate carboxylase; RB and LB, the right and left border sequences; Tet-on, the system to drive gene expression. (IV) The editing efficiency of Cre-*loxP* in filamentous fungi.

mechanism in cells, thus facilitating the editing of genome target sites. Compared to ZFNs, the occurrence rate of off-target effects for TALENs is relatively lower due to their longer recognition sequences, better design, and open-source capabilities. At the same time, TALENs also encounter challenges due to extensive identical repeat sequences. To address these issues, various methods have been developed, including Golden Gate cloning,[56] high-throughput solid-phase assembly,[57] and ligation-independent cloning techniques.[58] However, no examples of ZFNs and TALENs application in filamentous fungi have been found until now, properly due to the complicated design and large size of the fused protein used in ZFNs and TALENs.[59]

2.3 | CRISPR/Cas tool

The typical CRISPR-Cas9 system consists of a CRISPR sequence, the Cas9 gene, and a guide RNA (gRNA) sequence. CRISPR is a repetitive sequence in the genome of prokaryotes, with a length of approximately 21–47 bp. It is mainly composed of repeated sequences and spacer sequences. Among them, the leading sequence is located at upstream of CRISPR and is considered the promoter of the CRISPR sequence.[60,61] Transcripts of the repeated sequences can form hairpin structures, contributing to the stabilization of the overall RNA secondary structure. Spacer sequences are foreign DNA sequences captured by bacteria. The Cas gene is mainly responsible for coding nuclease, DNA helicase, polymerase, and other proteins. Guided by the single-stranded RNA (sgRNA), the Cas protein can cleave the target site and achieve DNA cleavage.

Due to its advantages in easy gene manipulation and specific targeting, the CRISPR-Cas system has been increasingly applied in various model and non-model filamentous fungi in recent years.[62] Genome editing techniques based on the CRISPR-Cas system for filamentous fungi include insertion, deletion, base conversion, and transcriptional activation at specific sites. The length of editing can be changed by one base, or it can be a gene cluster with a deletion.[1] Furthermore, by interrupting key genes of the host NHEJ and designing exquisite HR donors,[63] the CRISPR-Cas system can introduce changes at specific locations, enhancing editing accuracy.

2.4 | NgAgo tool

Argonaute proteins are a family of endonucleases that bind and cleave 5′ phosphorylated short single-stranded DNA. Similar to Cas9, argonautes play a key role in repressing gene expression and defending against exogenous nucleic acids.[64] However, the question of whether NgAgo (argonautes from *Natronobacterium gregory*) can act as a DNA-guided nucleic acid endonuclease for targeted cleavage of DNA in eukaryotes has been long debated.[65] Previous studies have shown that NgAgo can bind to target genes to inhibit the transcription process of the target genes and reduce the expression level of the target genes, but a reportdenied that NgAgo has a DNA cleavage function or a DNA editing function.[66] Nonetheless, some studies have purified NgAgo and tested its activity and function in vitro, with experimental results showing that the soluble and non-refolded structure of the NgAgo molecule has a random DNA-cutting function in vitro.[67,68] However, NgAgo has a clear disadvantage – it can only cut DNA with negative superhelical conformation and does not have cutting activity for linearized double-stranded DNA and ssDNA. As more argonautes, including PfAgo, MbpAgo, and others, are explored, these proteinscould potentially become gene editing tools in filamentous fungi and find applications in various gene manipulation areas.

2.5 | Fanzor tool

Recently, Feng Zhang's team discovered a novel gene editing technology known as the Fanzor system, which is an RNA-guided DNA cleavage system similar to the popular CRISPR-Cas system.[69] The Fanzor system represents the first programmable RNA-guided system found in eukaryotes, including animals, and is also the first CRISPR-like system found in eukaryotes. The authors conducted biochemical characterization of the Fanzor protein, demonstrating its programmability to recognize and cleave specific sequences on target DNA under RNA guidance. Moreover, the Fanzor system has no paracrine cleavage activity; it does not ripple through neighboring DNA when cutting specific DNA, minimizing unintended editing effects. With further optimization, the Fanzor system is expected to become a more precise and easy-to-deliver gene editing tool for filamentous fungi than existing technologies. Although RNA-guided Fanzor endonuclease exhibits bright prospects in the biological field, the specific mechanism of its activity remains unclear, which directly leads to uncertainty in evaluating its biosafety.[70] In terms of gene editing efficiency, compared to the widely known CRISPR-Cas9 system, the efficiency of Fanzor technology is slightly low,[71] thus its practical application in biological organisms still requires further development and optimization. As Fanzor is an emerging gene editing technology, there are still many issues to be addressed in terms of toxicity and applicability, which means that further exploration and validation are needed before applying it to future gene therapy for diseases.[72]

3 | CRISPR-CAS SYSTEM-MEDIATED GENOME EDITING TECHNOLOGY IN FILAMENTOUS FUNGI

The CRISPR/Cas system has become a key gene editing technology in the biological field due to its ease of operation and high editing efficiency.[73] And the CRISPR-Cas system offers numerous advantages for functional genomics research in filamentous fungi.[74]However, the low probability of HR in filamentous fungi makes them prone to the random integration of foreign DNA at unintended genomic loci, resulting in the off-target phenomenon.[75] At the same time, filamentous fungi lack certain promoters necessary for expressing sgRNA, making it difficult for the CRISPR-Cas9 system to proceed smoothly in filamentous fungi.[29] Consequently, researchers have reasonably adjusted the CRISPR-Cas system, forming a stan-

dardized set of CRISPR editing processes for filamentous fungi. This adaptation has sparked significant interest in applying this gene editing system in filamentous fungi (Figure 3).[1,76] However, studies on how to improve the efficiencies of the CRISPR/Cas system have been ongoing in filamentous fungi.

3.1 | Strategies to enhance gene editing in filamentous fungi

The CRISPR-Cas9 system can achieve genome-level genetic modifications through either NHEJ or homologous recombination repair (HDR) mechanisms. Both sgRNA and the Cas9 protein are critical factors for successful genome editing.[77,78]

Selecting appropriate promoters is essential to drive the efficient transcription of sgRNA, and then realize genome editing. Currently, there are two main strategies for sgRNA transcription: in vitro transcription and in vivo transcription mediated by RNA polymerase II or RNA polymerase III promoter.[79-82] For in vivo sgRNA transcription, numerous RNA polymerase III promoters were confirmed and characterized. Song et al.[63] tested 37 tRNA promoters for sgRNA transcriptions in *A. niger*, 36 of which can drive the transcription of sgRNA to achieve genome editing. However, in some instances, the efficiency of genome editing was found to be extremely low. Similarly, Zheng et al.[83] tested three different sources of U6 promoters and observed significant differences in sgRNA transcription levels driven by these promoters. Nevertheless, all these sgRNAs effectively functioned for CRISPR/Cas systems in *A. niger*. In contrast, in vitro transcription generally involves the synthesis of sgRNA and co-transformation with a Cas expression plasmid into the host for genome editing.[84] This approach does not require a promoter to drive sgRNA expression, and the presence of the RNP complex reduces sgRNA degradation. In addition, the integration of exogenous genes into the genome can be avoided by delivering the RNP complex into the host cell.[85,86]

The Cas protein with endonuelease function is a critical component of the CRISPR-Cas9 system. Codon optimization and selection of a suitable promoter can optimize Cas protein expression and thus influence gene editing efficiency.[87,88] The approach of codon optimization has been successfully constructed not only in *Trichoderma reesei*,[89] but also in other filamentous fungi such as *Pyricularia oryzae* and *Ganoderma lucidum*.[90] In general, the codon optimization methods for Cas proteins can be classified into bacterial codon optimization,[89] fungal codon optimization,[88] and human codon optimization.[31] Notably, for some filamentous fungi, the human codon-optimized Cas version works more effectively.[91] In addition to selecting strong endogenous promoters to increase the efficiency of CRISPR-Cas9-mediated gene editing, a recent study found that introducing introns in the Cas protein could also improve genome editing efficiency in *G. lucidum*.[92] Researchers speculate that the presence of introns may contribute to the accumulation of exogenous mRNA in fungi,[93] thereby enhancing the expression of the Cas protein. Furthermore, the enhanced expression of Cas9 would be beneficial for improving the genome editing efficiencies of the CRISPR-Cas system and reducing off-target effects. However, the expression of Cas9 as a foreign gene in a strain may be have drawbacks.[94] Therefore, in order to ensure the normal functioning of the CRISPR system in filamentous fungi, it is crucial to evaluate the potential differences in growth, metabolism, and generational stability of Cas-expressing strains.[95,96]

3.2 | CRISPR-Cas technology in filamentous fungi

With more and more in-depth studies focusing on the gene manipulations of filamentous fungi, CRISPR-Cas9 technology has been widely used in filamentous fungi. As shown in Table 1, the CRISPR-Cas system not only dramatically improves the efficiency of gene editing of various model strains such as *A. nidulans* and *T. reesei* but also realizes the precise gene editing of non-model strains such as *G. lucidum* that are difficult to be genetically manipulated.

Simultaneously, the CRISPR system is being complemented by a growing array of emerging technologies, encompassing the CRISPR single base editor, multiple base editor, and CRISPR/Cas12a. Drawing on the targeting capabilities of the CRISPR-Cas system, Huang et al.[97] developed a single-base editor for *A. niger*. Specifically, researcherscombined non-cleaving nCas9 or dCas9 with rat cytosine deaminase rAPOBEC1, enabling the conversion of cytosine to thymine in CRISPR-Cas-recognized target sequences. The efficiencies of editing report genes *pyrG* and *fwnA* using this editor ranged from 47.36% to 100%, while the efficiency of editing non-report gene *prtT* was 60%, suggesting a possible correlation with the protospacer. The system not only simplifies the investigation of gene function in *A. niger* but also introduces a novel approach to genetic manipulation in filamentous fungi. In order to explore new natural products, Zhao et al.[98] developed a multi-site editing system based on CRISPR base editor in filamentous fungi, enabling simultaneous inactivation of multiple genes. Scientistsfirst established a cytosine base editing technique (CBE), which could mutate specific positions of cytosine into thymine. According to this principle, mutations in the codons CAG, CAA, CGA, and CCA of the antisense chain of the target gene could lead to the insertion of termination codons into the gene sequence, early termination of transcription, and gene inactivation. The CBE and tRNA-gRNA cassette expression systems were co-transformed into fungal protoplasts to achieve genome editing, with statistical results showing that the average genome editing efficiency of the system was 67.4%. Subsequently, the researchers used the multi-site base editor to manipulate multiple epigenetic transcription factors in *A. nidulans*, among which multiple novel natural products were identified in the LO8030 mutant strain. *A. niger* is an important industrial microorganism, and its products such as organic acids and industrial enzymes are widely used in food, medicine, and chemical industries. To improve the screening efficiency of positive colonies on the primary transformation plate, Li et al.[99] designed a tRNA-gRNA array that targets both pigment genes and target genes, making the edited *A. niger* easily identifiable. The author first observed the association with the color of transformant colonies by knocking out the *albA*, *pptA*, and *brnA* pigment genes, and found that knocking out

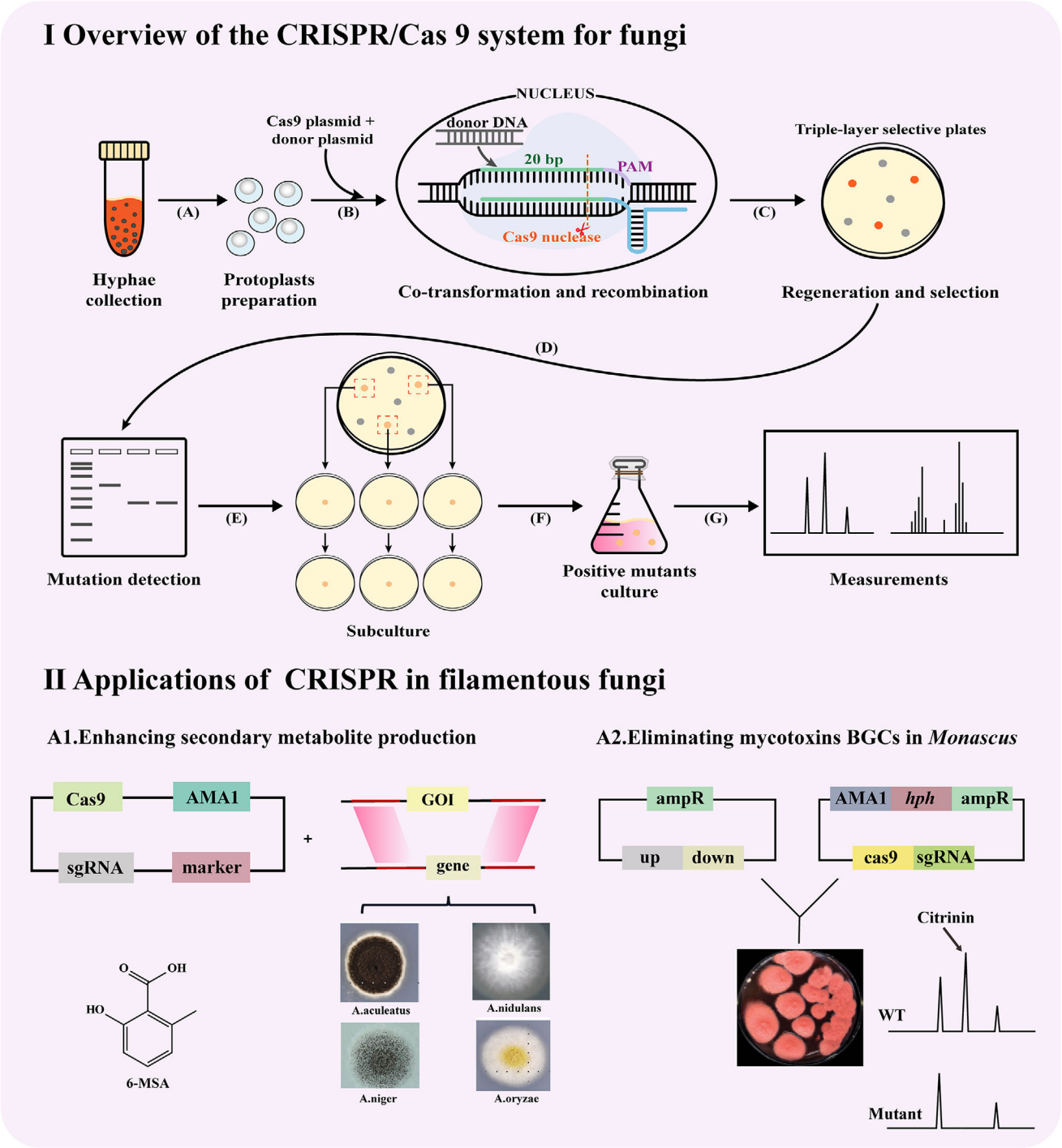

FIGURE 3 Operation process and applications of CRISPR-Cas9 tools in filamentous fungi. (I) Overview of the CRISPR/Cas 9 system for fungi. (A) Collecting young hyphae and obtaining protoplasts by enzymolysis. (B) Co-transforming protoplasts with donor plasmid and Cas9 plasmid using different genetic transformations to accomplish precise gene deletion. (C) After the incubation period, distributing the protoplast-containing mixture onto triple-layer selective agar plates to promote regeneration. (D) The transformants need to be verified through genomic PCR extraction. (E) Serial subculturing the positive transformants, extracting their genomic DNA, and conducting PCR verification. (F) Validating the correct transformants through shake flask fermentation. (G) Processing the samples appropriately and verifying the yield using various detection methods. (II) Applications of CRISPR in filamentous fungi: (A1) Heterologous expression of genes of interest (GOI) in a new strain to improve the yield of secondary metabolites, such as 6-methylsalicylic acid (6-MSA).[194] (A2) Utilization of a dual-plasmid CRISPR/Cas system for mycotoxin elimination in *Monascus*.

TABLE 1 Development of CRISPR/Cas9 system in filamentous fungi.

Strain	Delivery method	Expression strategy	Editing method and efficiency
Magnaporthe oryzae	Co-culture of RNP with protoplasts using calcium phosphate mineralized nanoparticles	Expression and assembly of functional RNP in vitro.	NHEJ, the efficiency of interrupting the *sdh* gene encoding syntalone dehydratase is 20%.[188]
Aspergillus niger	PEG-mediated protoplast transformation	The plasmid that can be replicated and lost is loaded with CRISPR-Cas system, and gRNA is expressed in the form of RNA polymerase type II promoter plus ribozyme.	Two transformants with correct integration form were obtained per 1 μg of DNA.[189]
Aspergillus niger	PEG-mediated protoplast transformation	Cas9 and endogenous 5S rRNA gene promoter optimized by *Aspergillus niger* codon drives gRNA expression.	HDR, efficiency is 33.3%–100%.[82]
Aspergillus fumigatus	PEG-mediated protoplast transformation	Endogenous expression of human codon optimized Cas9 and in vitro transcription of gRNA.	HDR, efficiency is 95%–100%.[31]
Trichoderma reesei	PEG-mediated protoplast transformation	Endogenous expression of *Trichoderma reesei* optimized *Cas9* and in vitro transcriptional mature gRNA.	HDR, the efficiency of editing an endogenous putative methyltransferase gene is 93%.[89]
Aspergillus oryzae	NM[a]	Transcription and expression of codon optimized Cas9 gene using *amyB* strong promoter and terminator.	NHEJ, 1 bp insertion or 1–21 bp deletion mutation is generated at the target site, and the editing efficiency is 10%–100%.[190]
Monascus ruber	PEG-mediated protoplast transformation	Dual single-guide RNAs were constructed to inactivate *MpigI* and *MpigI'*.	Six putative mutant strains were selected from 35 transformants.[191]
Monascus pilosus	PEG-mediated protoplast transformation	codon-optimized *Cas9* gene from *Streptococcus pyogenes* and the sgRNA is embedded in the middle of a larger transcript synthesized by RNA polymerase II.	RT-qPCR test confirmed the successful constructions of site-specific gene editing strains, the gene replacement frequencies were 27.4‰ for *mpclr4*, 37.5‰ for *mpdot1*, and 33.3‰ for *mplig4*.[104]
Ganoderma lucidum	PEG-mediated protoplast transformation	Gene disruption of *ura3* by codon-optimized *Cas9* and in vitro transcribed gRNAs.	NM[a][192]

HDR, homology-directed repair; NHEJ, non-homologous end joining.
[a]Not mentioned in the original text.

the *albA* gene resulted in white colonies. They optimized the position of the pigment gene *albA* and the promoter and terminator of the array to obtain the optimal tRNA-gRNA array. Finally, to test the knockout efficiencies of the system, the *ammA*, *amyA*, *prtT*, *kusA*, and *glaA* genes were knocked out, with the gene destruction rates in the white transformants reaching 89.2%, 70.91%, 50%, 22.41%, and 4.17%, respectively. Following the conclusive demonstration of the endonuclease activity of Cas12a, the CRISPR-Cas12a system has been increasingly used in the studies of filamentous fungi due to its excellent high specificity as well as multibase editing capabilities.[100,101] For instance, researchers have successfully established the CRISPR/Cas12a genome editing technology in *Myceliophthora thermophila*.[102] When utilizing this technology to simultaneously edit multiple genes within the genome, the editing efficiency for single gene knockout can attain a remarkable 100%, while the efficiency for dual-gene editing falls within the range of 61%–69%. Furthermore, the Mortense and coworkers made significant progress by successfully editing the *yA* gene in *Aspergillus tectonicus* and the *albA* gene in *A. niger*,[103] leveraging the Cpf1/CRISPR-tRNA expression system and *pyrG* screening markers. Notably, the research also achieved the integration of red fluorescent proteins into the respective loci of *yA* and *albA*, further demonstrating the versatility and precision of CRISPR-Cas12a in filamentous fungi research.

Although CRISPR gene editing technology has been used less frequently in *Monascus*, gene editing studies in *Monascus* is expected to progress rapidly given its efficient editing ability in other *Aspergillus* and the optimization of the *Aspergillus* transformation system.[104] These emerging technologies have been applied in some filamentous fungi, their applications are still relatively limited and have not yet achieved widespread popularity. If other emerging technologies, such as CRISPR/Cas13/14 gene editing system, prime editors, and CRISPR screening, can be successfully introduced and applied to filamentous fungi, it will undoubtedly simplify the gene editing process of filamentous fungi, which will help us to understand the growth and metabolism mechanism of filamentous fungi and open up new paths for scientific research and technological application in this field.

4 | CITRININ REMOVAL AND METABOLITE BIOSYNTHESIS IN *Monascus*

Different from yeast, filamentous fungi's varying nucleus numbers across hyphae and spores hinder genome editing advancements. *Monascus* typically relies on random mutagenesis for industrial CIT reduction, yet mutant strains display genetic instability, risking CIT production restoration. The difficulty also limits the large-scale production of *Monascus* products. To further expand the application area of *Monascus*, *Monascus*' engineering for eliminating toxic mycotoxins and enhancing their bioactive ingredients holds significant research value. At the same time, due to the involvement of polyketide synthases in the biosynthetic pathways of functional compounds in *Monascus*, we have also compared the modular organization of the functional domains identified in PKSs (Figure 4). Additionally, we have conducted an appropriate discussion on the impact of synthetic biology on *Monascus*.

4.1 | Citrinin

CIT, a polyketide mycotoxin produced by *Penicillium* and *Aspergillus* during growth, induces toxic effects on animals and humans through oxidative stress.[105] Therefore, the existence of mycotoxins as a global problem has raised widespread concern in the international community.[106,107] At present, there are many ways to reduce the content of CIT involves using molecular biology methods to knock out the genes responsible for CIT synthesis,[108,109] adding some flavonoids to the fermentation process,[110,111] increasing the culture medium,[112] and using low-frequency magnetic field (LF-MF) to altering metabolism.[113]

A comprehensive and systematic study of CIT in *Monascus* was conducted using gene knockout and heterologous expression strategies.[114] This led to the proposal of a model for the biosynthetic pathway, revealing that PKS catalyzes the synthesis of CIT skeleton through the condensation of malonyl-CoA and acetyl-CoA, which is then modified by *CitA-E* to complete the CIT synthesis (Figure 4).

Researchers have successfully utilized HR to knock out individual genes for the CIT biosynthesis pathway.[109,115–117] The approach led to the generation of mutant strains, which resulted in reduced CIT production to a certain extent but did not completely eliminate CIT production. Shimizu et al.[118] cloned the region of the 21 kb PKS encoding CIT polyketide synthase from *Monascus purpureus*, and it was found that there were four open reading frames (*orf1*, *orf2*, *orf3*, and *orf4*) in the 5′ region and an additional *orf5* in the 3′ region. Among them, *orf2* encodes a 576 amino acid protein with a typical Zn (II)2Cys6 DNA binding motif at the N-terminus, named *ctnA*. By using the autonomously replicated plasmid, the complementary effects for *ctnA* interference and overexpression on CIT production showed different results, indicating that *ctnA* is a key gene responsible for CIT biosynthesis. Additionally, the *orf6* gene, located between *ctnE* and *orf1*, encodes proteins similar to glyoxalase, and its function has been elucidated through the successful construction of *orf6* knockout strains, demonstrating its direct involvement in CIT biosynthesis of *M. purpureus* YY-1.[119] The *ctnB* gene encoding oxidoreductase has been to be reported to locate between *pksCT* and *ctnA*. A study has successfully used a pCTNB-HPH vector for the replacement of *ctnB* gene with hygromycin resistance genes as selective markers.[116] And the results show that the production of CIT was reduced. In recent years, Liang et al.[115] suggested that the CIT synthesis might have evolved genes or isoenzymes of *pksCT* by analyzing the transcriptomic levels between *M. purpureus* YY-1 and its mutant with low CIT production strain. Researchers have developed a genome editing system for industrial multinuclear microorganisms that utilizes a dual plasmid system with Cas9 and homologous recombinase plasmids to achieve markerless deletion of large DNA fragments.[1] By introducing resistance genes and a polykaryotic replicon into the Cas9 plasmid, this method efficiently and tracelessly deletes the CIT biosynthesis gene cluster.

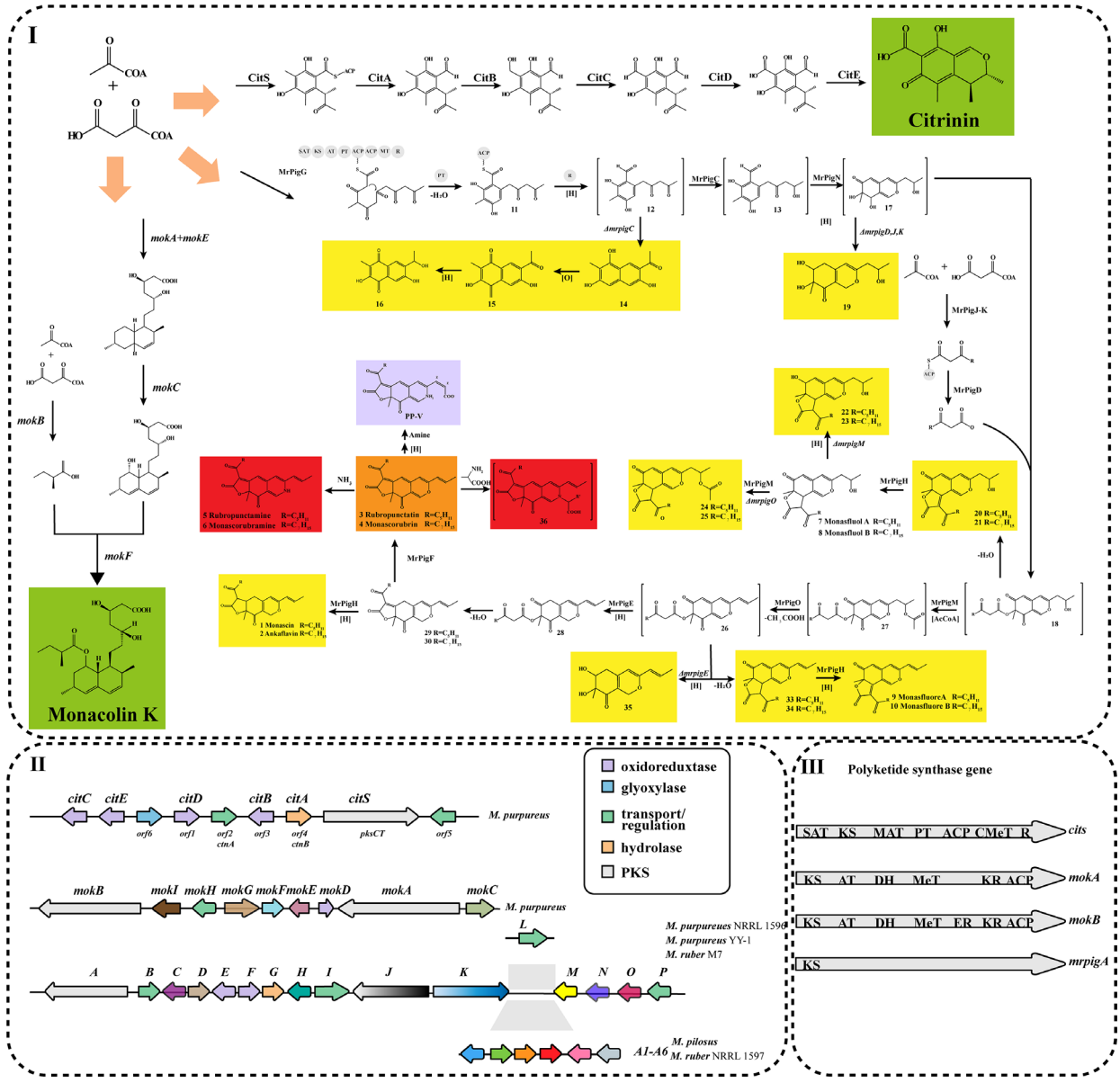

FIGURE 4 Biosynthesis of citrinin, *Monascus* pigments and Monacolin K. (I) The biosynthetic pathways of citrinin, *Monascus* pigments and Monacolin K. Yellow pigments: 1, 2, 13–17, 19–25, 29, 30, and 33–35. Orange pigments: 3 and 4. Red pigments: 5, 6, and 36. PP-V: violet pigment. (II) Schematic representation of the Citrinin, Monacolin K and *Monascus* pigments BGC. (III) Arrangement of functional domains of genes encoding polyketide synthases (PKSs) in the biosynthesis of citrinin, *Monascus* pigments and Monacolin K. DH, dehydratase; KR, keto-reductase; KS, ketosynthase family; MAT, malonyl-CoA: ACP transacylase domain; MeT, methyltransferase; PT, product template domain; SAT, N-terminal acyl carrier protein transacylase domain.

And the LC-MS analysis revealed that no CIT was detectd for the mutant strains after 10 rounds of cultivation, while red pigment yield increased by 5% compared to the wild type.

4.2 | Lovastatin

Lovastatin, also known as Monacolin K (MK), an active component in *Monascus* fermentation products,[120–122] inhibits cholesterol synthesis in the liver by competitively binding to HMG-CoA reductase, resulting in lower blood cholesterol and LDL-C levels.[46] Additionally, due to its inhibition of HMG-CoA reductase activity, which affects the cellular mevalonate pathway, and its ability to induce cellular changes, MK is also capable of preventing and treating colon cancer, acute leukemia, and brain tumors.[123] At present, there are many ways to improve the content of MK involving using molecular biology methods to improve the expression of key genes of MK synthesis,[123] optimizing the medium compositions and X-ray irradiation to enhance the production.[124]

MK is mainly produced by two filamentous fungi, *Monascus* and *Aspergillus terreus*. Chen et al.[125] isolated the MK synthase gene cluster of *Monascus* by constructing a cDNA library and preliminarily

identified the function of each gene in the gene cluster, confirming that the biosynthesis path of MK in *Monascus* is consistent with that in *A. terreus* (Figure 4).

Zhang et al.[126] successfully obtained mutant strains by overexpressing MK synthesis-related genes including *mokC*, *mokD*, *mokE*, and *mokI*, demonstrating that increasing the copy numbers of these genes effectively enhances MK production. Moreover, the overexpression strain of dual genes (*mokC* and *mokD*) in *Monascus pilosus* achieved in 54.85% increment in the yield of lovastatin, when compared with the start strain GN-01.[127] When the expression level of the global regulatory factor *LaeA* for secondary metabolites is increased, the MK production is enhanced by 1.5 times compared to the original strain.[128] The data of RT-qPCR shows that the transcription levels of *mokA*, *mokB*, *mokE*, and *mokH* in mutant strains were all increased, while the transcription levels of *mokC* and *mokD* were reduced. Meanwhile, some data suggested histone methylation modification plays an important role in regulating the biosynthesis of secondary metabolites in fungi.[129,130] Researchers constructed a deletion strain of the H3K9 methyltransferase, designated Δ*mpdot1*, in *M. pilosus* MS-1.[131] The subsequent results indicated that the strain produced lower levels of MK compared to the parental strain. After further analysis using RT-qPCR and Western blot, the researchers concluded that *mpdot1* influences lovastatin synthesis by regulating gene transcription and histone crosstalk.

4.3 | *Monascus* pigments

The main metabolites of *Monascus* also include MPs, which not only function as an edible pigment but also as an antioxidant, anticancer, antibacterial, anti-inflammatory, and anti-obesity substance.[132,133] *Monascus* can produce at least three shades of pigments, including red pigments (rubropunctamine and monascorubramine), orange pigments (rubropunctatin and monascorubrin), and yellow pigments (monascin and ankaflavin).[134] In ancient China, people preserved fish and meat with MPs, prized for its stability, safety, intense color, and low cost.[135,136] However, the synthesis pathway of MPs is similar to the pathway of CIT, and they show the requirement of the same acetyl-CoA and malonyl-CoA substance.[137,138] According to the document of European Union (Commission Regulation [EU] No 212/2014 of March 6, 2014), the food standards for MPs require the CIT titer to be less than or equal to 2 mg kg^{-1}. To enhance MPs production while minimizing CIT content, current research focuses on screening strains,[139,140] optimizing fermentation conditions,[141,142] and incorporating exogenous substances.[111,143] For example, with strain screening strategy, the MPs yield is almost two folds as compared to the parental strain and the CIT is almost non-existent.[140]

As illustrated in Figure 4, CIT and MPs share the majority of metabolic pathways. They are mainly generated through the fatty acid synthesis pathways using β-ketoic acid, combined with PKS-mediated carbon chain elongation of polyketide synthase. This process undergoes a *trans*-esterification reaction to produce orange pigments, which is then synthesized into yellow pigments and red pigments through reduction or amination, respectively.

Huang et al.[144] constructed *pksCT* gene deletion strains in *Monascus aurantiacus* to study pigment production by analyzing metabolites with GC-TOF-MS and establishing metabolic fingerprints. The deletion of *pksCT* resulted in a significant 98% reduction in CIT production, yet remarkably enhanced pigment yields. The phenomenon could be explained as the metabolic flux was shifted from acetyl-CoA to the TCA cycle in *pksCT* deletion strain, followed by metabolic flux switching to the biosynthetic polyketide pathway for the formation of pigments. ATP citrate lyase (ACL) plays a vital role in the formation of acetyl-CoA, which is a key precursor for the biosynthesis of MPs. The overexpression of ACLgenes significantly increased the activity of ACL and the content of acetyl-CoA, thereby increasing the production of MPs.[145,146] Overexpression of the global regulatory factor *LaeA* can affect mycelium growth and conidia formation, while MPs productions can also be improved.[128] In addition to global regulatory factors, the anabolism of filamentous fungi is also regulated by pathway-specific regulation. By constructing strains of *MPsGeI*, a pathway-specific regulator, Guo et al.[147] showed that *MPsGeI* negatively regulates the accumulation of MPs. Combined with transcriptome analysis, it was found that *MPsGeI* works by blocking the flow of high-concentration precursor substances to pigment synthesis and leads to the accumulation of large amounts of red pigments in mycelia. Studies have shown that exogenous addition of cyclic adenosine monophosphate (cAMP) can increase the yield of MPs.[148] In the metabolic pathway of cAMP, cAMP can be rapidly converted into 5′ adenine nucleoside by cAMP phosphodiesterases (PDEs). In theory, the concentration of cAMP can be increased by reducing or eliminating PDE activity, thereby regulating the production of secondary metabolites.[149] The study activated the cAMP signaling pathway by constructing a *mrPDE* knockout vector and obtained a high-yield yellow pigments *Monascus* strain.[150]

DMA, an antioxidant derived from *Monascus* solid-state fermentation (SSF), has undergone yield enhancement in red yeast rice primarily through the optimization of medium composition. And the production of DMA in red yeast rice is primarily determined by the expression levels of siderophore biosynthesis genes (*sidF*, *sidH*, *sidI*, and *sidD*) and transporter genes (*mirB*, *mirC*).[151] The fermentation metabolism of *Monascus* can also produce GABA. The strategy to improve GABA yield mainly lies in fermentation optimization,[152] and there are few studies on the modifications of its synthetic pathway. However, the key genes for GABA synthesis in *Monascus* YY-1 have already been identified.[153]

4.4 | Synthetic biology's dual impact on *monascus*

Gene-editing techniques can precisely help manipulate the *Monascus* genome to optimize its metabolites. For instance, CRISPR technology can help eliminate CIT,[1] thereby reducing the toxicity of *Monascus* products. Genetic modification can also enhance the growth rate and help produce beneficial metabolites of *Monascus*,[127,154] further boosting its industrial application.

However, biosafety is a non-negligible concern. Genetic modifications may lead *Monascus* to produce unknown metabolites,[155] which would take potential risks for the environment and human health. Therefore, rigorous biosafety assessments are required for modified strains before implementation. Additionally, establishing corresponding laws and regulations is essential to ensure the safe application of *Monascus*. Due to the precision and efficiency of genetic modifications, most strains may possess similar genetic backgrounds, leading to a decrease in genetic diversity within the *Monascus*. Successfully modified *Monascus* strains could potentially transfer exogenous genes to other microorganisms through horizontal gene transfer (HGT),[156] posing unknown risks and threats to the ecological environment.

5 | SOLID-STATE FERMENTATION: TRADITION, INNOVATION, AND FUTURE

The optimal environment for simulated strain growth was maximized by selecting different solid fermentation substrates to provide nitrogen sources, carbon sources, and nutrients.[157] Thus, SSF has wide applications in the production of enzymes, industrial chemicals, biofuels, and so on.[158-160] Currently, some filamentous fungi consortium systems can achieve co-metabolic activity under optimal co-culture conditions for SSF, efficiently utilizing the substrate and thus improving product purities. Although SSF is considered an important technology that can solve the energy crisis and environmental pollution, it also faces some problems such as poor heat and mass transfer, difficulties in controlling process parameters, and lack of efficient technology to realize large-scale production.

5.1 | Introduction to solid-state fermentation

At present, SSF is mainly used in the brewing of Baijiu, aged vinegar, etc. It uses natural substrates as carbon sources and energy sources, while wooden vats are usually used as solid supports. Its system is anhydrous or nearly anhydrous. The essence of Chinese Baijiu can be encapsulated in the succinct expression: good Chinese liquor comes from the SSF with pure grain. Pure grain means that the brewing process of Chinese Baijiu mainly uses grain as the main raw material, supplemented by distiller's yeast. From ancient to modern times, SSF has played a major role in the production of antibiotics, enzymes, refined feed, organic acids, bioactive substances, and further expanded to biotransformation, biofuels, biological control, garbage treatment and bioremediation, and other fields.[161] SSF can be used to biotransform wastes and by-products from different sources to improve their nutritional value and bring great economic value.[162] As an effective cell factory, SSF can increase the value of various hydrophilic and hydrophobic substrates and produce lipid-rich biological products. A study shows that *Mortierella alpina* can be used with SSF to biotransform animal fat by-products into high-value fermented biological products rich in arachidonic acid (ARA).[163] When animal fat by-products are mixed with corn flour in a certain proportion as substrates, the total fatty acid content of the fermented biological products is almost several times higher. To cope with the rapid rise of CO_2 in the atmosphere, it is necessary to seek fuel from renewable resources. Research has shown that white rot fungi can be used for value-added treatment of urban waste, achieving the potential for complete recycling of solid waste to produce mushrooms and bioethanol.[164] Cakes such as rapeseed meal, cottonseed meal, and castor bean meal are rich in high-quality protein, but their application in the aquaculture industry is limited due to the presence of toxic components such as gossypol, glucosinolates, tannins, or anti-nutritional factors. SSF using cakes for microorganism cultivation such as lactic acid bacteria, *Bacillus*, yeast, and filamentous fungi can effectively remove toxic components and degrade anti-nutritional factors.[165,166]

5.2 | Current status of SSF for *monascus* cultivation

Monascus SSF is a traditional fermentation technique that utilizes *Monascus* strains to produce MPs and other bioactive substances. Most SSF of red yeast still uses constant temperature star rooms, while some use shaking bottles. In recent years, *Monascus* SSF has garnered significant research interest, focusing on its promising applications in food, pharmaceutical, and nutritional supplement industries. In this sector, the current status of *Monascus* SSF and its comparison with conventional liquid fermentation will be discussed.

Recently, studies of *Monascus* SSF have focused on the following aspects: optimizing the formula and process conditions of fermentation substrate, conducting mechanism studies of the microbial community structures and their metabolic pathways to improve the product functionalities, and modifying the *Monascus* genome using gene editing technology to further improve the yields and qualities of *Monascus* SSF products. Among them, some interesting studies have been proposed to optimize the composition of SSF medium. The carbon source used for the industrial fermentation of *Monascus* spp. is generally rice or rice powders. By replacing the carbon source with yam powder, potato powder, cassava powder, sweet potato powder, etc., the yield of MK can be improved.[167,168] A type of renewable marine biomass (*Saccharina japonica*) was also reported to be used for *Monascus* SSF for lovastatin production.[169] Furthermore, different exogenous additives including glycerol, amino acids, etc., can also have a significant impact on *Monascus* SSF.[170,171]

Monascus SSF boasts advantages like simulating natural growth for product stability, promoting high-quality bioactives, and simplifying fermentation for cost savings,[170] but it also faces challenges and limitations compared to submerged fermentation. Firstly, *Monascus* SSF is currently limited to flask scale and is hard for large-scale fermentation. Secondly, moisture regulation and temperature control during SSF are difficult, which can easily lead to uneven fermentation processes and fluctuations in product quality. In addition, gas exchange and substrate supply during *Monascus* SSF are also issues of concern.

5.3 | Prospect of industrialization of solid-state fermentation of *monascus*

As a traditional fermentation technology that is also considered safe for producing products, *Monascus* SSF has broad development prospects for the future of the food industry and other fields. Taking into consideration the industrial standardization of *Monascus* SSF, it is necessary to further optimize the fermentation process and establish corresponding operation standards and product quality control standards. This involves replacing the traditional manual *Monascus* SSF using glass bottles, as multiple manual handling operations in SSF result in a fermentation process with high contamination rates and low productivity. To achieve this objective, control of process parameters such as fermentation temperature, humidity, and aeration conditions, as well as the monitoring and regulation of microbial communities during the fermentation process, are all necessary. At the same time, industrial applications also need to pay attention to product quality, safety, and regulatory supervision to ensure the sustainable development of the industry and the healthy development of the market.

In order to industrialize red yeast rice, there is an urgent need to solve the technical and economic problems arising from the fermentation process. Selecting a bioreactor suitable for industrial scale production and ensuring the quality of the fermented product are the two core issues to be faced at the technical level. One successful example of scale-up industrial SSF was carried out to produce the *Beauveria bassiana* for the development of mycoinsecticide,[172] which utilized polyethylene bags for SSF. In this case, a novel tray bioreactor with an atomization system for spore suspension, nutrient solution, and cooling water on a fermentation medium has been developed.[173] As a first successful industrial SSF example, it has been realized that the reactor can also be utilized to cultivate *Metarhizium anisopleliae* on a variety of different solid fermentation substrates. To realize precise control and monitoring of the fermentation process, and to improve the repeatability and production stability, the gas-phase dual-dynamic SSF equipment and the online detection system, such as near-infrared (NIR), tail gas GC-MS, etc., could be introduced to study the mechanism of the strain metabolism responding to the environmental changes during *Monascus* SSF, which would be very useful for *Monascus* industrializations. Based on these systems, the global dynamic regulation model for the *Monascus* SSF process and the precision fermentation control would be easily established, which would help the standardized control and the scale-up of industrial applications.

At the same time, in the process of industrialization and application, it is necessary to establish a corresponding quality control system and safety assessment methods to ensure that the products comply with food hygiene, safety, and regulatory standards. Finally, it is also necessary to pay close attention to aspects of market promotion and application expansion. Considering that the *Monascus* SSF products have a broad market prospect in the fields of food, drugs, and nutritional supplements, it is essential to strengthen market promotion and publicity to improve the consumer's knowledge and acceptance of the *Monascus* SSF products. Additionally, constant exploration of new application areas, such as cosmetics, and animal feeds, is also important. Of course, the costs also need to be considered in the industrialization process, which can be achieved by selecting energy-efficient automated control equipment to improve production efficiency and reduce energy consumption, as well as by optimizing raw material ratios, procurement channels or exploring low-cost raw materials. In addition, it can also seek government support and strengthen cooperation with universities to reduce enterprise pressure and improve technology.

In summary, with further studies on high-efficiency gene edition tools and equipment improvements, it is believed that *Monascus* SSF products will play a greater role in the food industry and are expected to expand to other fields, providing more possibilities for people's health and life. At the same time, the industrialized application also needs to pay attention to product quality, safety, and regulatory supervision to ensure the sustainable development of the industry and the healthy development of the market.

6 | PROSPECTS FOR A BRIGHT *MONASCUS* INDUSTRIAL FUTURE

With the continuous development and cross-integration of chemical biology, bioinformatics, synthetic biology, and natural product chemistry, the comprehensive development and utilization of natural products of *Monascus* fermentation will significantly promote the *Monascus* industry. Compared with other relatively mature filamentous fungal metabolic regulation systems that have been developed, there are still many shortcomings in using synthetic biology tools to improve the production of secondary metabolites of *Monascus*. To cope with the great challenges brought by the anabolism of filamentous fungi, we can combine the development trends of synthetic biology and process equipment enhancement. This allows us to progress from traditional breeding toward intelligent fermentation control (Figure 5).

To unlock the industrial potential application of *Monascus*, high-quality strains must be constructed. Developing efficient gene editing and multi-gene editing tools would facilitate the regulation of metabolic networks in *Monascus*, which breaks the bottleneck problems of mismatch between target pathway metabolism and cell physiological metabolism caused by single gene editing. To enhance the efficiency of CRISPR/Cas system in filamentous fungi, researchers devised new Cas proteins like Cas12k, Cas13, and Cpf1.[174] The combination of Cas12k and Tn7 transposable enzyme can accurately insert multiple DNA sequences into different sites of the target genome to achieve multi-site integration.[175] Currently, the combination of CRISPR/Cas systems with new technologies has led to the development of more effective gene editing tools. For example, Yin et al.[176] proposed a high-throughput sequencing method for primer extension-mediated sequencing (PEM-seq), which sensitively identifies off-target sites of Cas9 and quantifies the cutting efficiency of Cas9. Furthermore, single-cell RNA sequencing based on the CRISPR system can accurately explore gene function and genetic regulatory networks.[177]

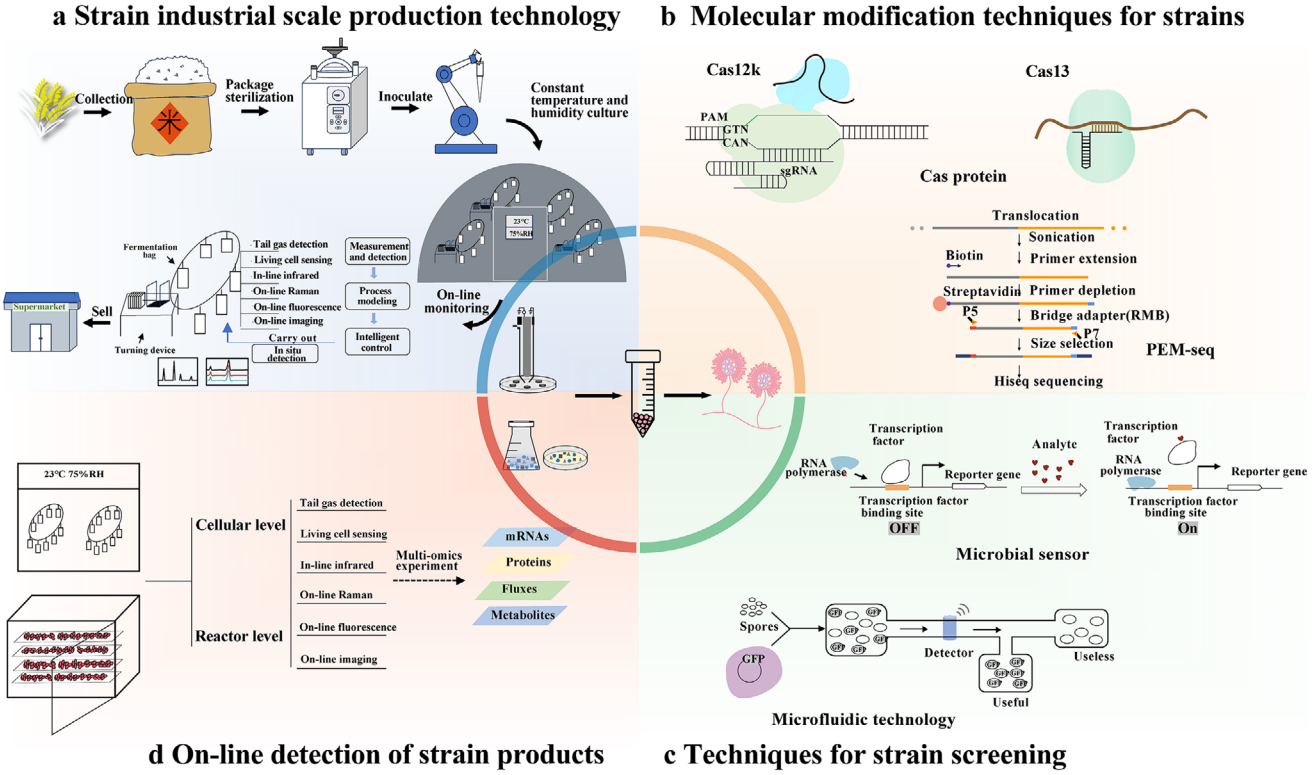

FIGURE 5 Perspective for the scale-up industrial production of *Monascus*.

Secondly, for efficient *Monascus* screening, high-throughput screening methods are needed. Bio-sensors can be screened from multiple perspectives such as transcription, translation, and protein activity, and can be used for high-throughput screening of high-yield strains for bioactive products. Luu et al.[178] developed a novel high-throughput screening method for gene transformation of filamentous fungi based on a droplet microfluidic system, in which the microfluidic platform screens recombinant strains based on the expression of green fluorescent protein (GFP). Zhu et al.[179] used colorimetry to successfully establish a biosensor that simultaneously detects two mycotoxins, and demonstrated good performance in aflatoxin B (AFB1) and ochratoxin A (OTA) detection. Regarding biosensors, various types of sensors have been developed, often for dynamic optimization of metabolic flux and efficient screening of desired phenotypic strains.[180,181]

Then, to ensure pollution-free and high-purity fermentation, a set of strain characterization and online product analysis technologies can be used for precise regulation and optimization. Microbial fermentation requires real-time monitoring and accurate quantification of cells, but because of the particularity of spores and mycelia of filamentous fungi, monitoring the fungal fermentation process becomes a major problem. One study designed an image analysis model, mass microscopic image intensity (MII), to predict the dry cell weight (DCW) during fermentation by analyzing the image intensity of mycelium collected by the microscope.[182] Additionally, a chemometric technique for quantifying protease activities in fungal SSF was established, which predicts biochemical parameters of metabolites based on the biochemical parameters and NIR spectral data using artificial neural network (ANN) and partial least squares (PLS) models.[42] Water activity (wa) is an important factor for SSF, Agarwal et al.[183] devised and authenticated an algorithm designed to accurately assess surface mass condensation, thereby holding the capability to refine and optimize aw during SSF. Utilizing real-time feedback from online monitoring devices, we can precisely adjust temperature and humidity to create the optimal environment for strain growth, and effectively reduce fermentation inconsistencies by altering the frequency of the turning device.

Finally, to improve the inefficient traditional SSF process in the *Monascus* SSF industry, an automatic SSF manufacturing line is vital to form a large-scale, automated, accurate intelligent SSF technology and efficient manufacturing integrated production process of *Monascus* SSF. As shown in Figure 5, with the comprehensive achievement of automatic turning and on-line monitoring devices, it would be easy to realize the industrial production for *Monascus* SSF using an intelligent integrated SSF process.

7 | CONCLUSION AND OUTLOOK

Biotechnological applications in genetic breeding or modification of filamentous fungi have effectively improved their capacity to synthesize bioactive substances. The CRISPR/Cas9 genome editing system has emerged as a superior alternative to traditional genome editing systems, being vigorously developed and applied in filamentous fungi. With a summarization of the development of the CRISPR/Cas9 system

in filamentous fungi gene manipulations, we can easily find the study directions for improving its editing efficiencies and reducing the off-target effect of the CRISPR/Cas9 system used in *Monascus* and other filamentous fungi.

At present, there are many genetic engineering methods for increasing MPs and MK production, including increasing precursor flux, overexpressing global regulatory factors, overexpressing key enzymes in the synthesis pathway, and inhibiting competitive pathways.[127,184] The synthesis of MK and MPs for *Monascus* is easily influenced by environmental factors, and researchers should focus on fermentation optimization.[185,186] Of course, due to the presence of CIT, there are significant safety issues with *Monascus* fermentation products. As long as the content of CIT is controlled within 2 mg kg^{-1}, it will not cause food safety issues. There are generally two strategies: knocking out related genes for CIT biosynthesis and optimizing fermentation parameters to reduce CIT contents.[108,168,187] Despite facing certain challenges in developing *Monascus* for industrial applications, advancements in synthetic biology technologies and industrial processes have paved the way for its exploration in various industries such as food, cosmetics, agriculture, medicine, and environmental protection. It is anticipated that in the near future, there will be a surge in the number of industries that explore the potential of *Monascus*.

AUTHOR CONTRIBUTIONS

Junping Zhou: Funding acquisition; conceptualization; formal analysis; investigation; writing—original draft; writing—review and editing. **Qilu Pan**: Investigation; writing—original draft; writing—review and editing. **Yinan Xue**: Investigation; writing—original draft. **Yaoing Dong**: Investigation; writing—original draft. **Yihong Chen**: Investigation; writing—original draft. **Lianggang Huang**: Project administration; supervision. **Bo Zhang**: Project administration; supervision. **Zhiqiang Liu**: Funding acquisition; conceptualization; project administration; supervision; writing—review and editing. **Yuguo Zheng**: Funding acquisition; conceptualization; project administration; supervision; writing—review and editing.

ACKNOWLEDGMENTS

This work was supported by the Program of the National Key Research and Development Program of China (2022YFA0911800) and Zhejiang Provincial Natural Science Foundation of China (LQ22C010001).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors do not have permission to share data.

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REFERENCES

1. Liu, W., An, C., Shu, X., Meng, X., Yao, Y., Zhang, J., Chen, F., Xiang, H., Yang, S., Gao, X., & Gao, S. S. (2020). A dual-plasmid CRISPR/Cas system for mycotoxin elimination in polykaryotic industrial fungi. *ACS Synthetic Biology*, *9*, 2087–2095. https://doi.org/10.1021/acssynbio.0c00178
2. Wang, Y. X., Yang, L., Xu, J. H., Xin, F. X., & Jiang, L. (2023). Applications of synthetic microbial consortia in biological control of mycotoxins and fungi. *Current Opinion in Food Science*, *53*, 101074. https://doi.org/10.1016/j.cofs.2023.101074
3. Molnar, Z., Bodai, V., Szakacs, G., Erdelyi, B., Fogarassy, Z., Safran, G., Varga, T., Konya, Z., Toth-Szeles, E., Szucs, R., & Lagzi, I. (2018). Green synthesis of gold nanoparticles by thermophilic filamentous fungi. *Scientific Reports*, *8*, 3943. https://doi.org/10.1038/s41598-018-22112-3
4. Mishra, A., Kumari, M., Pandey, S., Chaudhry, V., Gupta, K. C., & Nautiyal, C. S. (2014). Biocatalytic and antimicrobial activities of gold nanoparticles synthesized by *Trichoderma* sp. *Bioresource Technology*, *166*, 235–242. https://doi.org/10.1016/j.biortech.2014.04.085
5. Srikanth, M., Sandeep, T. S. R. S., Sucharitha, K., & Godi, S. (2022). Biodegradation of plastic polymers by fungi: A brief review. *Bioresources and Bioprocessing*, *9*, 42. https://doi.org/10.1186/s40643-022-00532-4
6. Cowan, A. R., Costanzo, C. M., Benham, R., Loveridge, E. J., & Moody, S. C. (2022). Fungal bioremediation of polyethylene: Challenges and perspectives. *Journal of Applied Microbiology*, *132*, 78–89. https://doi.org/10.1111/jam.15203
7. Wikandari, R., Hasniah, N., & Taherzadeh, M. J. (2022). The role of filamentous fungi in advancing the development of a sustainable circular bioeconomy. *Bioresource Technology*, *345*, 126531. https://doi.org/10.1016/j.biortech.2021.126531
8. Shi, Y. C., & Pan, T. M. (2011). Beneficial effects of *Monascus purpureus* NTU 568-fermented products: A review. *Applied Microbiology and Biotechnology*, *90*, 1207–1217. https://doi.org/10.1007/s00253-011-3202-x
9. Gong, P. F., Shi, R. Y., Liu, Y. T., Luo, Q. Q., Wang, C. T., & Chen, W. (2023). Recent advances in *monascus* pigments produced by *Monascus purpureus*: Biosynthesis, fermentation, function, and application. *LWT – Food Science and Technology*, *185*, 115162. https://doi.org/10.1016/j.lwt.2023.115162
10. Feng, Y., Shao, Y., & Chen, F. (2012). *Monascus* pigments. *Applied Microbiology and Biotechnology*, *96*, 1421–1440. https://doi.org/10.1007/s00253-012-4504-3
11. Mapari, S. A. S., Meyer, A. S., & Thrane, U. (2006). Colorimetric characterization for comparative analysis of fungal pigments and natural food colorants. *Journal of Agricultural and Food Chemistry*, *54*, 7027–7035. https://doi.org/10.1021/jf062094n
12. Zhang, Y., Chen, Z., Wen, Q., Xiong, Z., Cao, X., Zheng, Z., Zhang, Y., & Huang, Z. (2020). An overview on the biosynthesis and metabolic regulation of monacolin K/lovastatin. *Food & Function*, *11*, 5738–5748. https://doi.org/10.1039/d0fo00691b
13. Journoud, M., & Jones, P. J. (2004). Red yeast rice: A new hypolipidemic drug. *Life Sciences*, *74*, 2675–2683. https://doi.org/10.1016/j.lfs.2003.10.018
14. Yongxia, Z., Jian, X., Suyuan, H., Aixin, N., & Lihong, Z. (2020). Isolation and characterization of ergosterol from *Monascus anka* for anti-lipid peroxidation properties. *Journal De Mycologie Medicale*, *30*, 101038. https://doi.org/10.1016/j.mycmed.2020.101038
15. Tan, H., Xing, Z., Chen, G., Tian, X., & Wu, Z. (2018). Evaluating antitumor and antioxidant activities of yellow *Monascus* pigments from *Monascus ruber* fermentation. *Moleculars*, *23*, 3242. https://doi.org/10.3390/molecules23123242
16. (2018). Fungus focus. *Nature Ecology & Evolution*, *2*, 1675. https://doi.org/10.1038/s41559-018-0721-1
17. Alberti, F., Kaleem, S., & Weaver, J. A. (2020). Recent developments of tools for genome and metabolome studies in basidiomycete fungi and their application to natural product research. *Biology Open*, *9*, bio056010. https://doi.org/10.1242/bio.056010

18. Mohamed, H., Naz, T., Yang, J., Shah, A. M., Nazir, Y., & Song, Y. (2021). Recent molecular tools for the genetic manipulation of highly industrially important *Mucoromycota Fungi*. *Journal of Fungi*, *7*, 1061. https://doi.org/10.3390/jof7121061
19. Lichius, A., Ruiz, D. M., & Zeilinger, S. (2020). *Genetic transformation of filamentous fungi: Achievements and challenges*. Springer Publishing.
20. Wakai, S., Arazoe, T., Ogino, C., & Kondo, A. (2017). Future insights in fungal metabolic engineering. *Bioresource Technology*, *245*, 1314–1326. https://doi.org/10.1016/j.biortech.2017.04.095
21. Yin, K., & Qiu, J. L. (2019). Genome editing for plant disease resistance: Applications and perspectives. *Philosophical Transactions of the Royal Society B – Biological Sciences*, *374*, 20180322. https://doi.org/10.1098/rstb.2018.0322
22. McFarlane, G. R., Whitelaw, C. B. A., & Lillico, S. G. (2018). CRISPR-based gene drives for pest control. *Trends in Biotechnology*, *36*, 130–133. https://doi.org/10.1016/j.tibtech.2017.10.001
23. Karavolias, N. G., Horner, W., Abugu, M. N., & Evanega, S. N. (2021). Application of gene editing for climate change in agriculture. *Frontiers in Sustainable Food Systems*, *5*, 685801. https://doi.org/10.3389/fsufs.2021.685801
24. You, B. J., Lee, M. H., & Chung, K. R. (2009). Gene-specific disruption in the filamentous fungus *Cercospora nicotianae* using a split-marker approach. *Archives of Microbiology*, *191*, 615–622. https://doi.org/10.1007/s00203-009-0489-4
25. Goswami, R. S. (2012). Targeted gene replacement in fungi using a split-marker approach. *Methods in Molecular Biology*, *835*, 255–269. https://doi.org/10.1007/978-1-61779-501-5_16
26. Zhang, H. X., Zhang, Y., & Yin, H. (2019). Genome Editing with mRNA Encoding ZFN, TALEN, and Cas9. *Molecular Therapy*, *27*, 735–746. https://doi.org/10.1016/j.ymthe.2019.01.014
27. Shamshirgaran, Y., Liu, J., Sumer, H., Verma, P. J., & Taheri-Ghahfarokhi, A. (2022). Tools for efficient genome editing; ZFN, TALEN, and CRISPR. *Methods in Molecular Biology*, *2495*, 29–46. https://doi.org/10.1007/978-1-0716-2301-5_2
28. Shi, T. Q., Liu, G. N., Ji, R. Y., Shi, K., Song, P., Ren, L. J., Huang, H., & Ji, X. J. (2017). CRISPR/Cas9-based genome editing of the filamentous fungi: The state of the art. *Applied Microbiology and Biotechnology*, *101*, 7435–7443. https://doi.org/10.1007/s00253-017-8497-9
29. Shen, J. Y., Zhao, Q., & He, Q. L. (2023). Application of CRISPR in filamentous fungi and macrofungi: From component function to development potentiality. *ACS Synthetic Biology*, *12*, 1908–1923. https://doi.org/10.1021/acssynbio.3c00099
30. Liao, B., Chen, X., Zhou, X., Zhou, Y., Shi, Y., Ye, X., Liao, M., Zhou, Z., Cheng, L., & Ren, B. (2021). Applications of CRISPR/Cas gene-editing technology in yeast and fungi. *Archives of Microbiology*, *204*, 79. https://doi.org/10.1007/s00203-021-02723-7
31. Zhang, C., Meng, X., Wei, X., & Lu, L. (2016). Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. *Fungal Genetics and Biology*, *86*, 47–57. https://doi.org/10.1016/j.fgb.2015.12.007
32. Mei, Y. Z., Zhu, Y. L., Huang, P. W., Yang, Q., & Dai, C. C. (2019). Strategies for gene disruption and expression in filamentous fungi. *Applied Microbiology and Biotechnology*, *103*, 6041–6059. https://doi.org/10.1007/s00253-019-09953-2
33. Zhang, S., Ban, A., Ebara, N., Mizutani, O., Tanaka, M., Shintani, T., & Gomi, K. (2017). Self-excising Cre/mutant lox marker recycling system for multiple gene integrations and consecutive gene deletions in *Aspergillus oryzae*. *Journal of Bioscience and Bioengineering*, *123*, 403–411. https://doi.org/10.1016/j.jbiosc.2016.11.001
34. Kopke, K., Hoff, B., & Kuck, U. (2010). Application of the *Saccharomyces cerevisiae* FLP/FRT recombination system in filamentous fungi for marker recycling and construction of knockout strains devoid of heterologous genes. *Applied and environmental microbiology*, *76*, 4664–4674. https://doi.org/10.1128/AEM.00670-10
35. Kobayashi, K., Hattori, T., Honda, Y., & Kirimura, K. (2014). Oxalic acid production by citric acid-producing *Aspergillus niger* overexpressing the oxaloacetate hydrolase gene *oah*A. *Journal of Industrial Microbiology & Biotechnology*, *41*, 749–756. https://doi.org/10.1007/s10295-014-1419-2
36. Chib, S., Jamwal, V. L., Kumar, V., Gandhi, S. G., & Saran, S. (2023). Fungal production of kojic acid and its industrial applications. *Applied Microbiology and Biotechnology*, *107*, 2111–2130. https://doi.org/10.1007/s00253-023-12451-1
37. Chen, Y., Wang, J., Wang, M., Han, A., Zhao, X., Wang, W., & Wei, D. (2023). Engineering the metabolism and morphology of the filamentous fungus *Trichoderma reesei* for efficient L-malic acid production. *Bioresour Technology*, *387*, 129629. https://doi.org/10.1016/j.biortech.2023.129629
38. Yeh, H. H., Chiang, Y. M., Entwistle, R., Ahuja, M., Lee, K. H., Bruno, K. S., Wu, T. K., Oakley, B. R., & Wang, C. C. (2012). Molecular genetic analysis reveals that a nonribosomal peptide synthetase-like (NRPS-like) gene in *Aspergillus nidulans* is responsible for microperfuranone biosynthesis. *Applied Microbiology and Biotechnology*, *96*, 739–748. https://doi.org/10.1007/s00253-012-4098-9
39. Choi, S. Y., Lee, Y., Yu, H. E., Cho, I. J., Kang, M., & Lee, S. Y. (2023). Sustainable production and degradation of plastics using microbes. *Nature Reviews Microbiology*, *8*, 2253–2276. https://doi.org/10.1038/s41564-023-01529-1
40. Van Wylick, A., Rahier, H., De Laet, L., & Peeters, E. (2024). Conditions for $CaCO_3$ biomineralization by *Trichoderma Reesei* with the perspective of developing fungi-mediated self-healing concrete. *Global Challenges*, *8*, 2300160. https://doi.org/10.1002/gch2.202300160
41. Elsacker, E., Vandelook, S., & Peeters, E. (2023). Recent technological innovations in mycelium materials as leather substitutes: A patent review. *Frontiers in Bioengineering and Biotechnology*, *11*, 1204861. https://doi.org/10.3389/fbioe.2023.1204861
42. Barchi, A. C., Ito, S., Escaramboni, B., Neto, P. d. O., Herculano, R. D., Miranda, M. C. R., Passalia, F. J., Rocha, J. C., & Núñez, E. G. F. (2016). Artificial intelligence approach based on near-infrared spectral data for monitoring of solid-state fermentation. *Process Biochemistry*, *51*, 1338–1347. https://doi.org/10.1016/j.procbio.2016.07.017
43. Hang, X., Meng, L., Ou, Y., Shao, C., Xiong, W., Zhang, N., Liu, H., Li, R., Shen, Q., & Kowalchuk, G. A. (2022). *Trichoderma*-amended biofertilizer stimulates soil resident *Aspergillus* population for joint plant growth promotion. *NPJ Biofilms and Microbiomes*, *8*, 57. https://doi.org/10.1038/s41522-022-00321-z
44. Caspeta, L., & Nielsen, J. (2013). Toward systems metabolic engineering of *Aspergillus* and *Pichia* species for the production of chemicals and biofuels. *Biotechnology Journal*, *8*, 534–544. https://doi.org/10.1002/biot.201200345
45. Kuivanen, J., Penttila, M., & Richard, P. (2015). Metabolic engineering of the fungal D-galacturonate pathway for L-ascorbic acid production. *Microbial Cell Factories*, *14*, 2. https://doi.org/10.1186/s12934-014-0184-2
46. Lee, C. L., & Pan, T. M. (2012). Development of *Monascus* fermentation technology for high hypolipidemic effect. *Applied Microbiology and Biotechnology*, *94*, 1449–1459. https://doi.org/10.1007/s00253-012-4083-3
47. Konishi, H., Isozaki, S., Kashima, S., Moriichi, K., Ichikawa, S., Yamamoto, K., Yamamura, C., Ando, K., Ueno, N., Akutsu, H., Ogawa, N., & Fujiya, M. (2021). Probiotic *Aspergillus oryzae* produces anti-tumor mediator and exerts anti-tumor effects in pancreatic cancer through the p38 MAPK signaling pathway. *Scientific Reports*, *11*, 11070. https://doi.org/10.1038/s41598-021-90707-4
48. Bucholtz, F. (2008). Principles of site-specific recombinase (SSR) technology. *JoVE – Journal of Visualized Experiments*, 718. https://doi.org/10.3791/718
49. Shimshek, D. R., Kim, J., Hubner, M. R., Spergel, D. J., Buchholz, F., Casanova, E., Stewart, A. F., Seeburg, P. H., & Sprengel, R. (2002).

Codon-improved Cre recombinase (iCre) expression in the mouse. *Genesis (New York, N.Y.: 2000)*, *32*, 19–26. https://doi.org/10.1002/gene.10023
50. Parkitna, J. R., Engblom, D., & Schutz, G. (2009). Generation of Cre recombinase-expressing transgenic mice using bacterial artificial chromosomes. *Methods in Molecular Biology*, *530*, 325–342. https://doi.org/10.1007/978-1-59745-471-1_17
51. Park, Y. N., Masison, D., Eisenberg, E., & Greene, L. E. (2011). Application of the FLP/FRT system for conditional gene deletion in yeast *Saccharomyces cerevisiae*. *Yeast*, *28*, 673–681. https://doi.org/10.1002/yea.1895
52. Roux, I., & Chooi, Y. H. (2022). Cre/lox-mediated chromosomal integration of biosynthetic gene clusters for heterologous expression in *Aspergillus nidulans*. *ACS Synthetic Biology*, *11*, 1186–1195. https://doi.org/10.1021/acssynbio.1c00458
53. Wu, N., Wu, X., Zhang, M., Zhang, C., & Xu, Q. (2024). Metabolic engineering of *Aspergillus niger* for accelerated malic acid biosynthesis by improving NADPH availability. *Biotechnology Journal*, *19*, e2400014. https://doi.org/10.1002/biot.202400014
54. Steiger, M. G., Vitikainen, M., Uskonen, P., Brunner, K., Adam, G., Pakula, T., Penttila, M., Saloheimo, M., Mach, R. L., & Mach-Aigner, A. R. (2011). Transformation system for *Hypocrea jecorina* (*Trichoderma reesei*) that favors homologous integration and employs reusable bidirectionally selectable markers. *Applied and Environmental Microbiology*, *77*, 114–121. https://doi.org/10.1128/AEM.02100-10
55. Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., & Bonas, U. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science*, *326*, 1509–1512. https://doi.org/10.1126/science.1178811
56. Gaj, T., Gersbach, C. A., & Barbas, C. F., 3rd. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, *31*, 397–405. https://doi.org/10.1016/j.tibtech.2013.04.004
57. Briggs, A. W., Rios, X., Chari, R., Yang, L., Zhang, F., Mali, P., & Church, G. M. (2012). Iterative capped assembly: Rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. *Nucleic Acids Research*, *40*, e117. https://doi.org/10.1093/nar/gks624
58. Schmid-Burgk, J. L., Schmidt, T., Kaiser, V., Honing, K., & Hornung, V. (2013). A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. *Nature Biotechnology*, *31*, 76–81. https://doi.org/10.1038/nbt.2460
59. Wang, S., Chen, H., Tang, X., Zhang, H., Chen, W., & Chen, Y. Q. (2017). Molecular tools for gene manipulation in filamentous fungi. *Applied Microbiology and Biotechnology*, *101*, 8063–8075. https://doi.org/10.1007/s00253-017-8486-z
60. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*, 816–821. https://doi.org/10.1126/science.1225829
61. Hou, Z., & Zhang, Y. (2019). Inserting DNA with CRISPR. *Science*, *365*, 25–26. https://doi.org/10.1126/science.aay2056
62. Rozhkova, A. M., & Kislitsin, V. Y. (2021). CRISPR/Cas genome editing in filamentous fungi. *Biochemistry (Moscow)*, *86*, S120–S139. https://doi.org/10.1134/S0006297921140091
63. Song, L., Ouedraogo, J. P., Kolbusz, M., Nguyen, T. T. M., & Tsang, A. (2018). Efficient genome editing using tRNA promoter-driven CRISPR/Cas9 gRNA in *Aspergillus niger*. *PLoS ONE*, *13*, e0202868. https://doi.org/10.1371/journal.pone.0202868
64. Gao, F., Shen, X. Z., Jiang, F., Wu, Y., & Han, C. (2016). DNA-guided genome editing using the *Natronobacterium gregoryi* Argonaute. *Nature Biotechnology*, *34*, 768–773. https://doi.org/10.1038/nbt.3547
65. Burgess, S., Cheng, L., Gu, F., Huang, J., Huang, Z., Lin, S., Li, J., Li, W., Qin, W., Sun, Y., Songyang, Z., Wei, W., Wu, Q., Wang, H., Wang, X., Xiong, J. W., Xi, J., Yang, H., Zhou, B., & Zhang, B. (2017). Erratum to: Questions about NgAgo. *Protein & Cell*, *8*, 77. https://doi.org/10.1007/s13238-016-0349-3
66. Qi, J., Dong, Z., Shi, Y., Wang, X., Qin, Y., Wang, Y., & Liu, D. (2016). NgAgo-based *fabp11a* gene knockdown causes eye developmental defects in zebrafish. *Cell Research*, *26*, 1349–1352. https://doi.org/10.1038/cr.2016.134
67. Lee, K. Z., Mechikoff, M. A., Kikla, A., Liu, A., Pandolfi, P., Fitzgerald, K., Gimble, F. S., & Solomon, K. V. (2021). NgAgo possesses guided DNA nicking activity. *Nucleic Acids Research*, *49*, 9926–9937. https://doi.org/10.1093/nar/gkab757
68. Qu, J., Xie, Y., Guo, Z., Liu, X., Jiang, J., Chen, T., Li, K., Hu, Z., & Luo, D. (2021). Identification of a novel cleavage site and confirmation of the effectiveness of NgAgo gene editing on RNA targets. *Molecular Biotechnology*, *63*, 1183–1191. https://doi.org/10.1007/s12033-021-00372-1
69. Saito, M., Xu, P. Y., Faure, G., Maguire, S., Kannan, S., Altae-Tran, H., Vo, S., Desimone, A., Macrae, R. K., & Zhang, F. (2023). Fanzor is a eukaryotic programmable RNA-guided endonuclease. *Nature*, *620*, 660–668. https://doi.org/10.1038/s41586-023-06356-2
70. Yang, H., & Patel, D. J. (2024). Fanzors: Striking expansion of RNA-guided endonucleases to eukaryotes. *Cell Research*, *34*, 99–100. https://doi.org/10.1038/s41422-023-00894-0
71. Badon, I. W., Oh, Y., Kim, H. J., & Lee, S. H. (2024). Recent application of CRISPR-Cas12 and OMEGA system for genome editing. *Molecular Therapy*, *32*, 32–43. https://doi.org/10.1016/j.ymthe.2023.11.013
72. Zaib, S., Saleem, M. A., & Khan, I. (2022). CRISPR-Cas9 genome engineering: Trends in medicine and health. *Mini-Reviews in Medicinal Chemistry*, *22*, 410–421. https://doi.org/10.2174/1389557521666210913112030
73. Mishra, A., & Pandey, V. P. (2024). CRISPR/Cas system: A revolutionary tool for crop improvement. *Biotechnology Journal*, *19*, e2300298. https://doi.org/10.1002/biot.202300298
74. Deng, H., Gao, R., Liao, X., & Cai, Y. (2017). CRISPR system in filamentous fungi: Current achievements and future directions. *Gene*, *627*, 212–221. https://doi.org/10.1016/j.gene.2017.06.019
75. Shen, Q., Ruan, H., Zhang, H., Wu, T., Zhu, K., Han, W., Dong, R., Ming, T., Qi, H., & Zhang, Y. (2024). Utilization of CRISPR-Cas genome editing technology in filamentous fungi: Function and advancement potentiality. *Frontiers in Microbiology*, *15*, 1375120. https://doi.org/10.3389/fmicb.2024.1375120
76. Ullah, M., Xia, L., Xie, S., & Sun, S. (2020). CRISPR/Cas9-based genome engineering: A new breakthrough in the genetic manipulation of filamentous fungi. *Biotechnology and Applied Biochemistry*, *67*, 835–851. https://doi.org/10.1002/bab.2077
77. Mu, W., Zhang, Y., Xue, X., Liu, L., Wei, X., & Wang, H. (2019). 5' capped and 3' polyA-tailed sgRNAs enhance the efficiency of CRISPR-Cas9 system. *Protein Cell*, *10*, 223–228. https://doi.org/10.1007/s13238-018-0552-5
78. Garcia-Tunon, I., Alonso-Perez, V., Vuelta, E., Perez-Ramos, S., Herrero, M., Mendez, L., Hernandez-Sanchez, J. M., Martin-Izquierdo, M., Saldana, R., Sevilla, J., Sanchez-Guijo, F., Hernandez-Rivas, J. M., & Sanchez-Martin, M. (2019). Splice donor site sgRNAs enhance CRISPR/Cas9-mediated knockout efficiency. *PLoS ONE*, *14*, e0216674. https://doi.org/10.1371/journal.pone.0216674
79. Kleinstiver, B. P., Pattanayak, V., Prew, M. S., Tsai, S. Q., Nguyen, N. T., Zheng, Z. L., & Joung, J. K. (2016). High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*, *529*, 490–495. https://doi.org/10.1038/nature16526
80. Kuivanen, J., Wang, Y. J., & Richard, P. (2016). Engineering *Aspergillus niger* for galactaric acid production: Elimination of galactaric acid catabolism by using RNA sequencing and CRISPR/Cas9. *Microbial Cell Factories*, *15*, 210. https://doi.org/10.1186/s12934-016-0613-5
81. Song, L., Ouedraogo, J. P., Kolbusz, M., Nguyen, T. T. M., & Tsang, A. (2018). Efficient genome editing using tRNA promoter-driven

CRISPR/Cas9 gRNA in *Aspergillus niger*. *PLoS ONE*, *13*, e0202868. https://doi.org/10.1371/journal.pone.0202868

82. Zheng, X. M., Zheng, P., Zhang, K., Cairns, T. C., Meyer, V., Sun, J. B., & Ma, Y. H. (2019). 5S rRNA promoter for guide rna expression enabled highly efficient CRISPR/Cas9 genome editing in *Aspergillus niger*. *ACS Synthetic Biology*, *8*, 1568–1574. https://doi.org/10.1021/acssynbio.7b00456
83. Zheng, X., Zheng, P., Sun, J., Kun, Z., & Ma, Y. (2018). Heterologous and endogenous U6 snRNA promoters enable CRISPR/Cas9 mediated genome editing in *Aspergillus niger*. *Fungal Biology and Biotechnology*, *5*, 2. https://doi.org/10.1186/s40694-018-0047-4
84. Kuivanen, J., Arvas, M., & Richard, P. (2017). Clustered genes encoding 2-keto-l-gulonate reductase and l-idonate 5-dehydrogenase in the novel fungal d-glucuronic acid pathway. *Frontiers in Microbiology*, *8*, 225. https://doi.org/10.3389/fmicb.2017.00225
85. Zou, G., Xiao, M., Chai, S., Zhu, Z., Wang, Y., & Zhou, Z. (2021). Efficient genome editing in ilamentous fungi via an improved CRISPR-Cas9 ribonucleoprotein method facilitated by chemical reagents. *Microbial Biotechnology*, *14*, 2343–2355. https://doi.org/10.1111/1751-7915.13652
86. Zuris, J. A., Thompson, D. B., Shu, Y., Guilinger, J. P., Bessen, J. L., Hu, J. H., Maeder, M. L., Joung, J. K., Chen, Z. Y., & Liu, D. R. (2015). Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing *in vitro* and *in vivo*. *Nature Biotechnology*, *33*, 73–80. https://doi.org/10.1038/nbt.3081
87. Sugano, S. S., Suzuki, H., Shimokita, E., Chiba, H., Noji, S., Osakabe, Y., & Osakabe, K. (2017). Genome editing in the mushroom-forming basidiomycete *Coprinopsis cinerea*, optimized by a high-throughput transformation system. *Scientific Reports*, *7*, 1260. https://doi.org/10.1038/s41598-017-00883-5
88. Nodvig, C. S., Nielsen, J. B., Kogle, M. E., & Mortensen, U. H. (2015). A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS ONE*, *10*, e0133085. https://doi.org/10.1371/journal.pone.0133085
89. Liu, R., Chen, L., Jiang, Y., Zhou, Z., & Zou, G. (2015). Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. *Cell Discovery*, *1*, 15007. https://doi.org/10.1038/celldisc.2015.7
90. Arazoe, T., Miyoshi, K., Yamato, T., Ogawa, T., Ohsato, S., Arie, T., & Kuwata, S. (2015). Tailor-made CRISPR/Cas system for highly efficient targeted gene replacement in the rice blast fungus. *Biotechnology and Bioengineering*, *112*, 2543–2549. https://doi.org/10.1002/bit.25662
91. Tong, Y., Weber, T., & Lee, S. Y. (2019). CRISPR/Cas-based genome engineering in natural product discovery. *Natural Product Reports*, *36*, 1262–1280. https://doi.org/10.1039/c8np00089a
92. You, H., Sun, B., Li, N., & Xu, J. W. (2021). Efficient expression of heterologous genes by the introduction of the endogenous glyceraldehyde-3-phosphate dehydrogenase gene intron 1 in *Ganoderma lucidum*. *Microbial Cell Factories*, *20*, 164. https://doi.org/10.1186/s12934-021-01654-8
93. Laxa, M. (2016). Intron-mediated enhancement: A tool for heterologous gene expression in plants? *Frontiers in Plant Science*, *7*, 1977. https://doi.org/10.3389/fpls.2016.01977
94. Miyagishi, M., & Taira, K. (2002). U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nature Biotechnology*, *20*, 497–500. https://doi.org/10.1038/nbt0502-497
95. Schuster, M., Schweizer, G., Reissmann, S., & Kahmann, R. (2016). Genome editing in *Ustilago maydis* using the CRISPR-Cas system. *Fungal Genetics and Biology*, *89*, 3–9. https://doi.org/10.1016/j.fgb.2015.09.001
96. Deng, H., Gao, R., Liao, X., & Cai, Y. (2017). Genome editing in *Shiraia bambusicola* using CRISPR-Cas9 system. *Journal of Biotechnology*, *259*, 228–234. https://doi.org/10.1016/j.jbiotec.2017.06.1204
97. Huang, L., Dong, H., Zheng, J., Wang, B., & Pan, L. (2019). Highly efficient single base editing in *Aspergillus niger* with CRISPR/Cas9 cytidine deaminase fusion. *Microbiological Research*, *223–225*, 44–50. https://doi.org/10.1016/j.micres.2019.03.007
98. Zhao, F. L., Sun, C. X., Liu, Z. W., Cabrera, A., Escobar, M., Huang, S. Y., Yuan, Q. C., Nie, Q. Y., Luo, K. L., Lin, A. E. L., Vanegas, J. A., Zhu, T., Hilton, I. B., & Gao, X. (2023). Multiplex base-editing enables combinatorial epigenetic regulation for genome mining of fungal natural products. *Journal of the American Chemical Society*, *145*, 413–421. https://doi.org/10.1021/jacs.2c10211
99. Li, C., Zhou, J., Rao, S., Du, G., & Liu, S. (2021). Visualized multigene editing system for *Aspergillus niger*. *ACS Synthetic Biology*, *10*, 2607–2616. from: https://doi.org/10.1021/acssynbio.1c00231
100. Paul, B., & Montoya, G. (2020). CRISPR-Cas12a: Functional overview and applications. *Biomedical Journal*, *43*, 8–17. https://doi.org/10.1016/j.bj.2019.10.005
101. Katayama, T., & Maruyama, J. I. (2022). CRISPR/Cpf1-mediated mutagenesis and gene deletion in industrial filamentous fungi *Aspergillus oryzae* and *Aspergillus sojae*. *Journal of Bioscience and Bioengineering*, *133*, 353–361. https://doi.org/10.1016/j.jbiosc.2021.12.017
102. Liu, Q., Zhang, Y., Li, F., Li, J., Sun, W., & Tian, C. (2019). Upgrading of efficient and scalable CRISPR-Cas-mediated technology for genetic engineering in thermophilic fungus *Myceliophthora thermophila*. *Biotechnology for Biofuels and Bioproducts*, *12*, 293. https://doi.org/10.1186/s13068-019-1637-y
103. Vanegas, K. G., Jarczynska, Z. D., Strucko, T., & Mortensen, U. H. (2019). Cpf1 enables fast and efficient genome editing in *Aspergilli*. *Fungal Biology and Biotechnology*, *6*, 6. https://doi.org/10.1186/s40694-019-0069-6
104. Gong, Y., Li, S., Liu, Q., Chen, F., & Shao, Y. (2024). CRISPR/Cas9 system is a suitable gene targeting editing tool to filamentous fungus *Monascus pilosus*. *Applied Microbiology and Biotechnology*, *108*, 154. https://doi.org/10.1007/s00253-023-12865-x
105. Wu, J., Yang, C., Yang, M., Liang, Z., Wu, Y., Kong, X., Fan, H., Wang, S., Ning, C., Xiao, W., Jin, Y., Yi, J., & Yuan, Z. (2022). The role of ER stress and ATP/AMPK in oxidative stress meditated hepatotoxicity induced by citrinin. *Ecotoxicology and Environmental Safety*, *237*, 113531. https://doi.org/10.1016/j.ecoenv.2022.113531
106. Turner, P. C., Flannery, B., Isitt, C., Ali, M., & Pestka, J. (2012). The role of biomarkers in evaluating human health concerns from fungal contaminants in food. *Nutrition Research Reviews*, *25*, 162–179. https://doi.org/10.1017/S095442241200008x
107. Schmidt, J., Cramer, B., Turner, P. C., Stoltzfus, R. J., Humphrey, J. H., Smith, L. E., & Humpf, H. U. (2021). Determination of urinary mycotoxin biomarkers using a sensitive online solid phase extraction-UHPLC-MS/MS method. *Toxins*, *13*, 418. https://doi.org/10.3390/toxins13060418
108. Fu, G., Xu, Y., Li, Y., & Tan, W. (2007). Construction of a replacement vector to disrupt *pksCT* gene for the mycotoxin citrinin biosynthesis in *Monascus aurantiacus* and maintain food red pigment production. *Asia Pacific Journal of Clinical Nutrition*, *16*(Suppl 1), 137–142. https://doi.org/CorpusID.3963826.
109. Ning, Z. Q., Cui, H., Xu, Y., Huang, Z. B., Tu, Z., & Li, Y. P. (2017). Deleting the citrinin biosynthesis-related gene, *ctnE*, to greatly reduce citrinin production in *Monascus aurantiacus* Li AS3.4384. *International Journal of Food Microbiology*, *241*, 325–330. https://doi.org/10.1016/j.ijfoodmicro.2016.11.004
110. Ouyang, W., Liu, X., Wang, Y., Huang, Z., & Li, X. (2021). Addition of genistein to the fermentation process reduces citrinin production by *Monascus via* changes at the transcription level. *Food Chemistry*, *343*, 128410. https://doi.org/10.1016/j.foodchem.2020.128410
111. Wang, Y., Gao, H., Xie, J., Li, X., & Huang, Z. (2020). Effects of some flavonoids on the mycotoxin citrinin reduction by *Monascus aurantiacus* Li AS3.4384 during liquid-state fermentation. *AMB Express*, *10*, 26. https://doi.org/10.1186/s13568-020-0962-7

112. Zhou, B., Wang, J., Pu, Y., Zhu, M., Liu, S., & Liang, S. (2009). Optimization of culture medium for yellow pigments production with *Monascus anka* mutant using response surface methodology. *European Food Research and Technology*, *228*, 895–901. https://doi.org/10.1007/s00217-008-1002-z
113. Wan, Y., Zhang, J., Han, H., Li, L., Liu, Y., & Gao, M. (2016). Citrinin-producing capacity of *Monascus purpureus* in response to low-frequency magnetic fields. *Process Biochemistry*, *53*, 25–29. https://doi.org/10.1016/j.procbio.2016.11.009
114. He, Y., & Cox, R. J. (2016). The molecular steps of citrinin biosynthesis in fungi. *Chemical Science*, *7*, 2119–2127. https://doi.org/10.1039/c5sc04027b
115. Liang, B., Du, X. J., Li, P., Sun, C. C., & Wang, S. (2018). Investigation of citrinin and pigment biosynthesis mechanisms in *Monascus purpureus* by transcriptomic analysis. *Frontiers in Microbiology*, *9*, 1374. https://doi.org/10.3389/fmicb.2018.01374
116. Li, Y. P., Pan, Y. F., Zou, L. H., Xu, Y., Huang, Z. B., & e, Q. H. (2013). Lower citrinin production by gene disruption of *ctnB* involved in citrinin biosynthesis in *Monascus aurantiacus* Li AS3.4384. *Journal of Agricultural and Food Chemistry*, *61*, 7397–7402. https://doi.org/10.1021/jf400879s
117. Li, Y. P., Tang, X., Wu, W., Xu, Y., Huang, Z. B., & He, Q. H. (2015). The *ctnG* gene encodes carbonic anhydrase involved in mycotoxin citrinin biosynthesis from *Monascus aurantiacus*. *Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment*, *32*, 577–583. https://doi.org/10.1080/19440049.2014.990993
118. Shimizu, T., Kinoshita, H., & Nihira, T. (2007). Identification and *in vivo* functional analysis by gene disruption of *ctnA*, an activator gene involved in citrinin biosynthesis in *Monascus purpureus*. *Applied and Environmental Microbiology*, *73*, 5097–5103. https://doi.org/10.1128/AEM.01979-06
119. Liang, B., Du, X., Li, P., Guo, H., Sun, C., Gao, J., & Wang, S. (2017). *Orf6* gene encoded glyoxalase involved in mycotoxin citrinin biosynthesis in *Monascus purpureus* YY-1. *Applied Microbiology and Biotechnology*, *101*, 7281–7292. https://doi.org/10.1007/s00253-017-8462-7
120. Klingelhofer, I., & Morlock, G. E. (2019). Lovastatin in lactone and hydroxy acid forms and citrinin in red yeast rice powders analyzed by HPTLC-UV/FLD. *Analytical and Bioanalytical Chemistry*, *411*, 6655–6665. https://doi.org/10.1007/s00216-019-02039-y
121. Patakova, P. (2013). *Monascus* secondary metabolites: Production and biological activity. *Journal of Industrial Microbiology & Biotechnology*, *40*, 169–181. https://doi.org/10.1007/s10295-012-1216-8
122. Verhoeven, V., Lopez Hartmann, M., Remmen, R., Wens, J., Apers, S., & Van Royen, P. (2013). Red yeast rice lowers cholesterol in physicians—A double blind, placebo controlled randomized trial. *BMC Complementary Medicine and Therapies*, *13*, 178. https://doi.org/10.1186/1472-6882-13-178
123. Xiong, Z., Cao, X., Wen, Q., Chen, Z., Cheng, Z., Huang, X., Zhang, Y., Long, C., Zhang, Y., & Huang, Z. (2019). An overview of the bioactivity of monacolin K /lovastatin. *Food and Chemical Toxicology*, *131*, 110585. https://doi.org/10.1016/j.fct.2019.110585
124. Ye, F., Zhang, C., Liu, S., Liu, X., Liu, J., Guo, T., Lu, D., & Zhou, X. (2024). Optimization of medium compositions and X-ray irradiation to enhance monacolin K production by *Monascus purpureus* in submerged fermentation. *Process Biochemistry*, *141*, 50–60. https://doi.org/10.1016/j.procbio.2024.03.006
125. Chen, W. P., He, Y., Zhou, Y. X., Shao, Y. C., Feng, Y. L., Li, M., & Chen, F. S. (2015). Edible filamentous fungi from the species *Monascus*: Early traditional fermentations, modern molecular biology, and future genomics. *Comprehensive Reviews in Food Science and Food Safety*, *14*, 555–567. https://doi.org/10.1111/1541-4337.12145
126. Zhang, C., Liang, J., Zhang, A., Hao, S., Zhang, H., Zhu, Q., Sun, B., & Wang, C. (2019). Overexpression of Monacolin K biosynthesis genes in the *Monascus purpureus* azaphilone polyketide pathway. *Journal of Agricultural and Food Chemistry*, *67*, 2563–2569. https://doi.org/10.1021/acs.jafc.8b05524
127. Hong, X., Guo, T., Xu, X., & Lin, J. (2023). Multiplex metabolic pathway engineering of *Monascus pilosus* enhances lovastatin production. *Applied Microbiology and Biotechnology*, *107*, 6541–6552. https://doi.org/10.1007/s00253-023-12747-2
128. Zhang, C., Zhang, H., Zhu, Q., Hao, S., Chai, S., Li, Y., Jiao, Z., Shi, J., Sun, B., & Wang, C. (2020). Overexpression of global regulator *LaeA* increases secondary metabolite production in *Monascus purpureus*. *Applied Microbiology and Biotechnology*, *104*, 3049–3060. https://doi.org/10.1007/s00253-020-10379-4
129. Zhang, J., Chen, Y., Wang, S., Liu, Y., Li, L., & Gao, M. (2023). Role of histone H3K4 methyltransferase in regulating *Monascus* pigments production by red light-coupled magnetic field. *Photochemistry and Photobiology*, *100*(1), 75–86. https://doi.org/10.1111/php.13809
130. Brosch, G., Loidl, P., & Graessle, S. (2008). Histone modifications and chromatin dynamics: A focus on filamentous fungi. *FEMS Microbiology Review*, *32*, 409–439. https://doi.org/10.1111/j.1574-6976.2007.00100.x
131. Gong, Y., Li, S., Zhou, Y., Chen, F., & Shao, Y. (2023). Histone lysine methyltransferases MpDot1 and MpSet9 are involved in the production of lovastatin and MonAzPs by histone crosstalk modification. *International Journal of Biological Macromolecules*, *255*, 128208. https://doi.org/10.1016/j.ijbiomac.2023.128208
132. Kurokawa, H., Ito, H., & Matsui, H. (2017). *Monascus purpureus* induced apoptosis on gastric cancer cell by scavenging mitochondrial reactive oxygen species. *Journal of Clinical Biochemistry and Nutrition*, *61*, 189–195. https://doi.org/10.3164/jcbn.17-27
133. Pasdaran, A., Zare, M., Hamedi, A., & Hamedi, A. (2023). A review of the chemistry and biological activities of natural colorants, dyes, and pigments: Challenges, and opportunities for food, cosmetics, and pharmaceutical application. *Chemistry & Biodiversity*, *20*, e202300561. https://doi.org/10.1002/cbdv.202300561
134. Chen, W. P., Feng, Y. L., Molnar, I., & Chen, F. S. (2019). Nature and nurture: Confluence of pathway determinism with metabolic and chemical serendipity diversifies azaphilone pigments. *Natural Product Reports*, *36*, 561–572. https://doi.org/10.1039/c8np00060c
135. Pailliè-Jiménez, M. E., Stincone, P., & Brandelli, A. (2020). Natural pigments of microbial origin. *Frontiers in Sustainable Food Systems*, *4*. https://doi.org/10.3389/fsufs.2020.590439
136. Antipova, T. V., Zhelifonova, V. P., Zaitsev, K. V., & Vainshtein, M. B. (2023). Fungal azaphilone pigments as promising natural colorants. *Microbiology*, *92*, 1–10. https://doi.org/10.1134/S0026261722601737
137. Qin, X. L., Xie, B., Zong, X. L., Yu, X., & Feng, Y. L. (2023). Selective production, relationship and controversy between *Monascus* pigments and citrinin. *Food Bioscience*, *56*, 103233. https://doi.org/10.1016/j.fbio.2023.103233
138. Pavesi, C., Flon, V., Mann, S., Leleu, S., Prado, S., & Franck, X. (2021). Biosynthesis of azaphilones: A review. *Natural Product Reports*, *38*, 1058–1071. https://doi.org/10.1039/d0np00080a
139. Ketkaeo, S., Sanpamongkolchai, W., Morakul, S., Baba, S., Kobayashi, G., & Goto, M. (2020). Induction of mutation in *Monascus purpureus* isolated from Thai fermented food to develop low citrinin-producing strain for application in the red koji industry. *The Journal of General and Applied Microbiology*, *66*, 163–168. https://doi.org/10.2323/jgam.2019.04.008
140. Dikshit, R., & Tallapragada, P. (2018). Development and screening of mutants from *Monascus sanguineus* for secondary metabolites production. *Beni-Suef University Journal of Basic and Applied Sciences*, *7*, 235–240. https://doi.org/10.1016/j.bjbas.2018.03.001
141. Hajjaj, H., Francois, J. M., Goma, G., & Blanc, P. J. (2012). Effect of amino acids on red pigments and citrinin production in *Monascus ruber*. *Journal of Food Science*, *77*, 156–159. https://doi.org/10.1111/j.1750-3841.2011.02579.x

142. Zhang, X., Liu, W., Chen, X., Cai, J., Wang, C., & He, W. (2017). Effects and mechanism of blue light on *Monascus* in liquid fermentation. *Molecules (Basel, Switzerland)*, *22*, 385. https://doi.org/10.3390/molecules22030385
143. Liu, H. Q., Huang, Z. F., Yang, S. Z., Tian, X. F., & Wu, Z. Q. (2021). Inducing red pigment and inhibiting citrinin production by adding lanthanum(III) ion in *Monascus purpureus* fermentation. *Applied Microbiology and Biotechnology*, *105*, 1905–1912. https://doi.org/10.1007/s00253-021-11162-9
144. Huang, Z., Zhang, S., Xu, Y., Li, L., & Li, Y. (2016). Metabolic effects of the *pksCT* gene on *Monascus aurantiacus* Li As3.4384 using gas chromatography–time-of-flight mass spectrometry-based metabolomics. *Journal of Agricultural and Food Chemistry*, *64*, 1565–1574. https://doi.org/10.1021/acs.jafc.5b06082
145. Long, C. N., Zeng, X., Xie, J., Liang, Y. M., Tao, J. J., Tao, Q. Q., Liu, M. M., Cui, J. J., Huang, Z. W., & Zeng, B. (2019). High-level production of *Monascus* pigments in *Monascus ruber* CICC41233 through ATP-citrate lyase overexpression. *Biochemical Engineering Journal*, *146*, 160–169. https://doi.org/10.1016/j.bej.2019.03.007
146. Shwab, E. K., & Keller, N. P. (2008). Regulation of secondary metabolite production in filamentous ascomycetes. *Mycological Research*, *112*, 225–230. https://doi.org/10.1016/j.mycres.2007.08.021
147. Guo, X., Atehli, D., Chen, M., Chen, D., & Wang, Y. (2023). A Zn(II)(2)Cys(6) transcription factor MPsGel suppresses pigment biosynthesis in *Monascus*. *International Journal of Biological Macromolecules*, *233*, 123504. https://doi.org/10.1016/j.ijbiomac.2023.123504
148. Lai, Y., Wang, L., Qing, L., & Chen, F. (2011). Effects of cyclic AMP on development and secondary metabolites of *Monascus ruber* M-7. *Letters in Applied Microbiology*, *52*, 420–426. https://doi.org/10.1111/j.1472-765X.2011.03022.x
149. Shimizu, K., & Keller, N. P. (2001). Genetic involvement of a cAMP-dependent protein kinase in a g protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics*, *157*, 591–600. https://doi.org/10.1093/genetics/157.2.591
150. Liu, J. W., Du, Y., Ma, H. M., Pei, X. L., & Li, M. (2020). Enhancement of *Monascus* yellow pigments production by activating the cAMP signalling pathway in *Monascus purpureus* HJ11. *Microbial Cell Factories*, *19*, 224. https://doi.org/10.1186/s12934-020-01486-y
151. Washim, K., Omkar, R., & Prasad, P. B. (2021). Enrichment of dimerumic acid in *Monascus*-fermented rice and its *in vivo* antioxidant activity. *Food Frontiers*, *2*, 547–556. https://doi.org/10.1002/fft2.108
152. Wu, A., Li, L., Zhang, S., Lin, Q., & Liu, J. (2023). Optimization of the hongqu starter preparation process for the manufacturing of red mold rice with high gamma-aminobutyric acid production by solid-state fermentation. *Biotechnology and Applied Biochemistry*, *70*, 458–468. https://doi.org/10.1002/bab.2370
153. Yue, Y., Bin, L., Xinjun, D., Ping, L., Bin, L., Xiaozhen, C., Liangcheng, D., Di, H., Lei, W., & Shuo, W. (2015). Complete genome sequence and transcriptomics analyses reveal pigment biosynthesis and regulatory mechanisms in an industrial strain. *Monascus purpureus* YY-1. *Scientific Reports*, *5*, 8331. https://doi.org/10.1038/srep08331
154. Zhang, J., Yang, Y., Mao, Z., Yan, Q., Chen, Q., Yi, M., & Shao, Y. (2022). Inactivation of *MrSir2* in *Monascus ruber* influenced the developmental process and the production of *Monascus* azaphilone pigments. *Applied Biochemistry and Biotechnology*, *194*, 5702–5716. https://doi.org/10.1007/s12010-022-04030-3
155. Huang, Z., Su, B., Xu, Y., Li, L., & Li, Y. (2017). Determination of two potential toxicity metabolites derived from the disruption of the *pksCT* gene in *Monascus aurantiacus* Li As3.4384. *Journal of the Science of Food and Agriculture*, *97*, 4190–4197. https://doi.org/10.1002/jsfa.8291
156. Khaldi, N., Collemare, J., Lebrun, M. H., & Wolfe, K. H. (2008). Evidence for horizontal transfer of a secondary metabolite gene cluster between fungi. *Genome Biology*, *9*, R18. https://doi.org/10.1186/gb-2008-9-1-r18
157. Egbune, E. O., Ezedom, T., Orororo, O. C., Egbune, O. U., Avwioroko, O. J., Aganbi, E., Anigboro, A. A., & Tonukari, N. J. (2023). Solid-state fermentation of cassava (Manihot esculenta Crantz): A review. *World Journal of Microbiology & Biotechnology*, *39*, 259. https://doi.org/10.1007/s11274-023-03706-0
158. Maehara, L., Pereira, S. C., Silva, A. J., & Farinas, C. S. (2018). One-pot strategy for on-site enzyme production, biomass hydrolysis, and ethanol production using the whole solid-state fermentation medium of mixed filamentous fungi. *Biotechnology Progress*, *34*, 671–680. https://doi.org/10.1002/btpr.2619
159. Shah, P. C., Kumar, V. R., Dastager, S. G., & Khire, J. M. (2017). Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. *AMB Express*, *7*, 66. https://doi.org/10.1186/s13568-017-0370-9
160. Cristina, C. A. A., Batista, M. D., Vieira, N. K. M., Graciano, d. P. R., Stecca, S. A. F. K. J., Ashok, P., & Kumar, G. V. N. S. R. (2022). Engineering of holocellulase in biomass-degrading fungi for sustainable biofuel production. *Journal of Cleaner Production*, *371*, 133488. https://doi.org/10.1016/j.jclepro.2022.133488
161. Singh, R., Das, R., Sangwan, S., Rohatgi, B., Khanam, R., Peera, S. K. P. G., Das, S., Lyngdoh, Y. A., Langyan, S., Shukla, A., Shrivastava, M., & Misra, S. (2021). Utilisation of agro-industrial waste for sustainable green production: A review. *Environmental Sustainability*, *4*, 619–636. https://doi.org/10.1007/s42398-021-00200-x
162. Chilakamarry, C. R., Mimi Sakinah, A. M., Zularisam, A. W., Sirohi, R., Khilji, I. A., Ahmad, N., & Pandey, A. (2022). Advances in solid-state fermentation for bioconversion of agricultural wastes to value-added products: Opportunities and challenges. *Bioresource Technology*, *343*, 126065. https://doi.org/10.1016/j.biortech.2021.126065
163. Slany, O., Klempova, T., Shapaval, V., Zimmermann, B., Kohler, A., & Certik, M. (2020). Biotransformation of animal fat-by products into ara-enriched fermented bioproducts by solid-state fermentation of *Mortierella alpina*. *Journal of Fungi*, *6*, 236. https://doi.org/10.3390/jof6040236
164. Xu, S., Gazo, M., Peng, Z. H., Sui, K. P., Li, Y., & Li, C. T. (2022). Upcycling from chitin-waste biomass into bioethanol and mushroom via solid-state fermentation with *Pleurotus ostreatus*. *Fuel*, *326*, 125061. https://doi.org/10.1016/j.fuel.2022.125061
165. Zhang, Z., Yang, D., Liu, L., Chang, Z., & Peng, N. (2022). Effective gossypol removal from cottonseed meal through optimized solid-state fermentation by *Bacillus coagulans*. *Microbial Cell Factories*, *21*, 252. https://doi.org/10.1186/s12934-022-01976-1
166. Li, J., Gao, T., Hao, Z., Guo, X., & Zhu, B. (2022). Anaerobic solid-state fermentation with *Bacillus subtilis* for digesting free gossypol and improving nutritional quality in cottonseed meal. *Frontiers in Nutrition*, *9*, 1017637. https://doi.org/10.3389/fnut.2022.1017637
167. Ignatius, S., Endang, K., Elok, Z., Susana, R., Ira, N., Andreas, A., Nathania, I., & Bo, Z. B. (2021). Utilization of agro-industrial by-products in *Monascus* fermentation: A review. *Bioresources and Bioprocessing*, *8*, 129. https://doi.org/10.1186/s40643-021-00473-4
168. Feng, Y., Shao, Y., Zhou, Y., & Chen, F. (2014). Production and optimization of monacolin K by citrinin-free *Monascus pilosus* MS-1 in solid-state fermentation using non-glutinous rice and soybean flours as substrate. *European Food Research and Technology*, *239*, 629–636. https://doi.org/10.1007/s00217-014-2259-z
169. Suraiya, S., Kim, J. H., Tak, J. Y., Siddique, M. P., Young, C. J., Kim, J. K., & Kong, I. S. (2018). Influences of fermentation parameters on lovastatin production by *Monascus* purpureus using *Saccharina japonica* as solid fermented substrate. *LWT – Food Science and Technology*, *92*, 1–9. https://doi.org/10.1016/j.lwt.2018.02.013
170. Zhang, B. B., Lu, L. P., & Xu, G. R. (2015). Why solid-state fermentation is more advantageous over submerged fermentation for converting high concentration of glycerol into Monacolin K by *Monascus*

purpureus 9901: A mechanistic study. *Journal of Biotechnology*, *206*, 60–65. https://doi.org/10.1016/j.jbiotec.2015.04.011

171. Zhang, C., Chai, S., Hao, S., Zhang, A., Zhu, Q., Zhang, H., & Wang, C. (2019). Effects of glutamic acid on the production of monacolin K in four high-yield monacolin K strains in *Monascus*. *Applied Microbiology and Biotechnology*, *103*, 5301–5310. https://doi.org/10.1007/s00253-019-09752-9
172. Pham, T. A., Kim, J. J., & Kim, K. (2010). Optimization of solid-state fermentation for improved conidia production of *Beauveria bassiana* as a mycoinsecticide. *Mycobiology*, *38*, 137–143. https://doi.org/10.4489/MYCO.2010.38.2.137
173. Dallastra, E. D. G., Dias, A. C. P., de Morais, P. B., da Silva, J. F. M., Casciatori, F. P., & Grajales, L. M. (2023). Development of a novel pilot-scale tray bioreactor for solid-state fermentation aiming at process intensification. *Chemical Engineering and Processing-Process Intensification*, *193*, 109526. https://doi.org/10.1016/j.cep.2023.109526
174. Perculija, V., Lin, J., Zhang, B., & Ouyang, S. (2021). Functional features and current applications of the RNA-targeting type VI CRISPR-Cas systems. *Advanced Science*, *8*, 2004685. https://doi.org/10.1002/advs.202004685
175. Yang, J. J., Yang, J. W., Zhang, Y. W., Yang, S. Q., Zhang, J. Z., Jiang, Y., & Yang, S. (2021). CRISPR-associated transposase system can insert multiple copies of donor DNA into the same target locus. *The CRISPR Journal*, *4*, 789–798. https://doi.org/10.1089/crispr.2021.0019
176. Yin, J. H., Liu, M. Z., Liu, Y., Wu, J. C., Gan, T. T., Zhang, W. W., Li, Y. H., Zhou, Y. X., & Hu, J. Z. (2019). Optimizing genome editing strategy by primer-extension-mediated sequencing. *Cell Discovery*, *5*, 18. https://doi.org/10.1038/s41421-019-0088-8
177. Replogle, J. M., Norman, T. M., Xu, A., Hussmann, J. A., Chen, J., Cogan, J. Z., Meer, E. J., Terry, J. M., Riordan, D. P., Srinivas, N., Fiddes, I. T., Arthur, J. G., Alvarado, L. J., Pfeiffer, K. A., Mikkelsen, T. S., Weissman, J. S., & Adamson, B. (2020). Combinatorial single-cell CRISPR screens by direct guide RNA capture and targeted sequencing. *Nature Biotechnology*, *38*, 954–961. https://doi.org/10.1038/s41587-020-0470-y
178. Luu, X. C., Shida, Y., Suzuki, Y., Sato, N., Nakumura, A., & Ogasawara, W. (2022). A novel high-throughput approach for transforming filamentous fungi employing a droplet-based microfluidic platform. *Nature Biotechnology*, *72*, 149–158. https://doi.org/10.1016/j.nbt.2022.11.003
179. Zhu, W., Li, L., Zhou, Z., Yang, X., Hao, N., Guo, Y., & Wang, K. (2020). A colorimetric biosensor for simultaneous ochratoxin A and aflatoxins B1 detection in agricultural products. *Food Chemistry*, *319*, 126544. https://doi.org/10.1016/j.foodchem.2020.126544
180. Diaz Nieto, C. H., Granero, A. M., Garcia, D., Nesci, A., Barros, G., Zon, M. A., & Fernandez, H. (2019). Development of a third-generation biosensor to determine sterigmatocystin mycotoxin: An early warning system to detect aflatoxin B(1). *Talanta*, *194*, 253–258. https://doi.org/10.1016/j.talanta.2018.10.032
181. Radwan, O., Brothers, M. C., Coyle, V., Chapleau, M. E., Chapleau, R. R., Kim, S. S., & Ruiz, O. N. (2022). Electrochemical biosensor for rapid detection of fungal contamination in fuel systems. *Biosensors & Bioelectronics*, *211*, 114374. https://doi.org/10.1016/j.bios.2022.114374
182. Lee, S. K., Lee, J. H., Kim, H. R., Chun, Y., Lee, J. H., Park, C., Yoo, H. Y., & Kim, S. W. (2021). Rapid and concise quantification of mycelial growth by microscopic image intensity model and application to mass cultivation of fungi. *Scientific Reports*, *11*, 24157. https://doi.org/10.1038/s41598-021-03512-4
183. Agarwal, V., Kumar, D., Varadwaj, P., & Tiwari, A. (2020). Water activity and biomass estimation using digital image processing in solid-state fermentation. *Bioresource Technology*, *308*, 123277. https://doi.org/10.1016/j.biortech.2020.123277
184. Duan, Y., Du, Y., Yi, Z., Wang, Z., Pei, X., Wei, X., & Li, M. (2022). Systematic metabolic engineering for the production of azaphilones in *Monascus purpureus* HJ11. *Journal of Agricultural and Food Chemistry*, *70*, 1589–1600. https://doi.org/10.1021/acs.jafc.1c07588
185. Jirasatid, S., Nopharatana, M., Kitsubun, P., Vichitsoonthonkul, T., & Tongta, A. (2013). Statistical optimization for Monacolin K and yellow pigment production and citrinin reduction by *Monascus purpureus* in solid-state fermentation. *Journal of Microbiology and Biotechnology*, *23*, 364–374. https://doi.org/10.4014/jmb.1206.06068
186. Embaby, A. M., Hussein, M. N., & Hussein, A. (2018). *Monascus* orange and red pigments production by *Monascus purpureus* ATCC16436 through co-solid state fermentation of corn cob and glycerol: An eco-friendly environmental low cost approach. *PLoS ONE*, *13*, e0207755. https://doi.org/10.1371/journal.pone.0207755
187. Chen, D., Xue, Y., Chen, M., Li, Z., & Wang, C. (2016). Optimization of submerged fermentation medium for citrinin-free monascin production by *Monascus*. *Preparative Biochemistry & Biotechnology*, *46*, 772–779. https://doi.org/10.1080/10826068.2015.1135461
188. Li, S. J., Song, Z. Y., Liu, C. Y., Chen, X. L., & Han, H. Y. (2019). Biomimetic mineralization-based CRISPR/Cas9 ribonucleoprotein nanoparticles for gene editing. *ACS Applied Materials & Interfaces*, *11*, 47762–47770. https://doi.org/10.1021/acsami.9b17598
189. Sarkari, P., Marx, H., Blumhoff, M. L., Mattanovich, D., Sauer, M., & Steiger, M. G. (2017). An efficient tool for metabolic pathway construction and gene integration for *Aspergillus niger*. *Bioresource Technology*, *245*, 1327–1333. https://doi.org/10.1016/j.biortech.2017.05.004
190. Katayama, T., Tanaka, Y., Okabe, T., Nakamura, H., Fujii, W., Kitamoto, K., & Maruyama, J. (2016). Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. *Biotechnology Letters*, *38*, 637–642. https://doi.org/10.1007/s10529-015-2015-x
191. Ree Yoon, H., Han, S., Chul Shin, S., Cheong Yeom, S., & Jin Kim, H. (2023). Improved natural food colorant production in the filamentous fungus *Monascus ruber* using CRISPR-based engineering. *Food Research International*, *167*, 112651. https://doi.org/10.1016/j.foodres.2023.112651
192. Wang, P. A., Xiao, H., & Zhong, J. J. (2020). CRISPR-Cas9 assisted functional gene editing in the mushroom *Ganoderma lucidum*. *Applied Microbiology and Biotechnology*, *104*, 1661–1671. https://doi.org/10.1007/s00253-019-10298-z
193. Xu, Y., Shan, L., Zhou, Y., Xie, Z., Ball, A. S., Cao, W., & Liu, H. (2019). Development of a Cre-loxP-based genetic system in *Aspergillus niger* ATCC1015 and its application to construction of efficient organic acid-producing cell factories. *Applied Microbiology and Biotechnology*, *103*, 8105–8114. https://doi.org/10.1007/s00253-019-10054-3
194. Jarczynska, Z. D., Rendsvig, J. K. H., Pagels, N., Viana, V. R., Nodvig, C. S., Kirchner, F. H., Strucko, T., Nielsen, M. L., & Mortensen, U. H. (2021). Diversity: A fungal multispecies gene expression platform. *ACS Synthetic Biology*, *10*, 579–588. https://doi.org/10.1021/acssynbio.0c00587

How to cite this article: Zhou, J., Pan, Q., Xue, Y., Dong, Y., Chen, Y., Huang, L., Zhang, B., Liu, Z., & Zheng, Y. (2024). Synthetic biology for *Monascus*: From strain breeding to industrial production. *Biotechnology Journal*, *19*, e2400180. https://doi.org/10.1002/biot.202400180