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Development of microbial chassis for production of fungal natural products

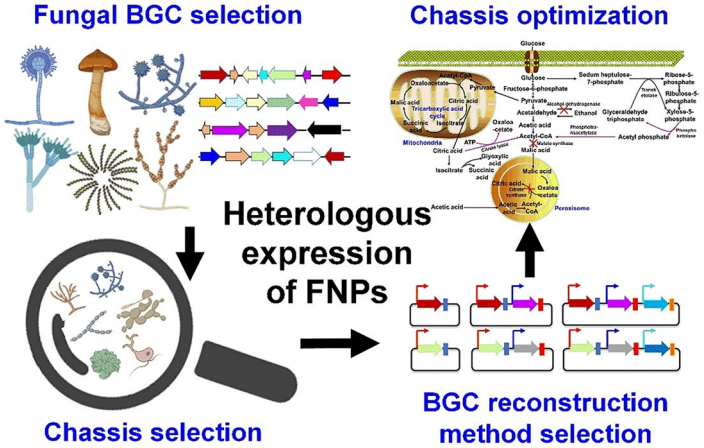
Junyang Wang[a,b], Xu Ji[a], Zhenlin Xin[a], Jinmiao Hu[a], Zihe Liu[a], and Shuobo Shi[a]

[a]Beijing Advanced Innovation Center for Soft Matter Science and Engineering, State Key Laboratory of Green Biomanufacturing, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China; [b]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

ABSTRACT

Fungal natural products (FNPs) are an important class of natural medicines. Genome sequencing has uncovered an unexpectedly large number of silent biosynthetic gene clusters (BGCs) in fungi that hold potential for FNP production. However, activating silent BGCs in native hosts is hindered by undesirable traits, such as difficulty in genetic manipulation or exhibiting extremely low metabolite titers. With the development of synthetic biology, heterologous expression systems are increasingly becoming the preferred option to overcome these limitations. In this review, we first summarize the major structural classes of FNPs and the corresponding backbone enzymes. We then evaluate the key features of various microbial chassis and strategies employed for pathway refactoring to achieve efficient heterologous expression. Furthermore, we discuss optimization strategies that enhance pathway flux toward the target product and minimize by-product formation. These methodologies are essential for advancing heterologous platforms for FNP discovery and biosynthesis. Additionally, we analyze current challenges and propose solutions to further improve microbial chassis for more effective FNP production.

GRAPHICAL ABSTRACT



STATEMENT OF SIGNIFICANCE

This review systematically compares prokaryotic, yeast, and filamentous fungal chassis for heterologous production of fungal natural products. It puts forward a "chassis–refactoring–compartmentalization" triad strategy to address silent gene cluster activation and low-titer bottlenecks, provides a roadmap from gram-scale discovery to kilogram-scale manufacturing, and fills the gap in design principles and scalability assessment of fungal natural product cell factories.

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Natural products; biosynthetic gene cluster; pathway refactoring; heterologous expression; genome streamlining; microbial chassis; synthetic biology

Introduction

The majority of fungal natural products (FNPs) are secondary metabolites that have undergone diverse structural and biological modifications over billions of years to adapt to their ecological niches and fulfill specific physiological functions [1,2]. These FNPs have not only been developed into therapeutic agents for human diseases, but have also contributed to advancements

CONTACT Shuobo Shi shishuobo@mail.buct.edu.cn; Zihe Liu zihe@mail.buct.edu.cn

in food production. Examples include cholesterol-lowering agents like lovastatin, immunosuppressants like cyclosporins, and plant growth hormones such as gibberellins and carotenoids.

Biosynthetic gene clusters (BGCs) responsible for FNPs have been extensively identified due to breakthroughs in genome sequencing technology. However, the discovery of corresponding FNPs has lagged behind expectations as approximately 90% of these BGCs remain "silent" under laboratory conditions [3,4]. Traditional methods to activate silent BGCs involve altering growth media, optimizing culture conditions, or employing microbial co-cultures, which can simultaneously activate multiple BGCs and facilitate the efficient discovery of fungal active natural products (NPs) with diverse structures. However, these approaches face increasing challenges, including the redundancy of known FNPs and difficulties in detecting low-yield compounds.

Recent attention has shifted toward genome mining rather than culture-dependent approaches. Notably, FNPs are typically synthesized by gene clusters located on chromosomes, often positioned near telomeres, which may facilitate efficient chemical skeleton synthesis, substrate transport, and regulatory control [5]. Core enzyme classes, such as: polyketide synthases (PKSs), non-ribosomal peptide synthases (NRPSs), terpene synthases/cyclases (TCs), and dimethylallyl tryptophan synthases (DMATSs), form the basis for characterizing BGCs and are used to classify FNPs according to their structural types. The Secondary Metabolite Unknown Regions Finder (SMURF) was the first comprehensive predictive toolkit specifically designed for fungal gene analysis, and it plays a pivotal role in identifying gene clusters associated with: NRPSs, PKSs, NRPS-PKS hybrids, indole alkaloids, and terpenoids [6]. Recently, bioinformatics tools for microbial genome mining have become increasingly widespread [7].

Genome mining efficiently identifies numerous BGCs involved in secondary metabolite biosynthesis through bioinformatics analysis. After identification, the activation of silent BGCs becomes critical. Emerging methods include modifying epigenetic pathways using small molecules such as DNA methyltransferases and histone deacetylase inhibitors to induce silenced gene expression [8]. Direct genetic manipulations in fungi, such as overexpression of positive regulators and promoter replacements, also stimulate BGC activation [1,9,10]. Additionally, modifying ribosomal proteins and transcription factors can activate previously silent antibiotic synthesis genes during stable growth phases [11]. However, many fungi exhibit undesirable traits for genetic manipulation.

For non-culturable or genetically inaccessible fungi, the exploration of FNPs involves integrating and expressing synthetic gene clusters of interest in heterologous hosts. Establishing a heterologous expression platform requires selecting: suitable chassis strains with a robust genetic background, advanced genetic manipulation tools, and efficient precursor supply. Cloning and transferring target BGCs into these hosts, along with metabolic engineering strategies such as pathway shunting and compartmentalization, can facilitate the detection of heterologously expressed FNPs even when present in low abundance.

Additionally, advancements in analytical tools, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, have significantly improved the efficiency of NP identification [7]. MS is favored for its sensitivity in analyzing complex metabolites, while NMR remains the gold standard for elucidating novel NP structures [7]. Recent breakthroughs in automated NMR spectral analysis and database development have further enhanced NP identification and classification [7]. These databases play a crucial role in NP research, and their construction has become an integral part of the discovery pipeline. In fact, over the last 20 years, more than 120 databases have been established, encompassing various types of NPs [12].

This paper focuses on the challenges associated with the discovery and efficient production of FNPs through heterologous expression of BGCs, along with corresponding solutions. Recent progress in the following areas will be critically reviewed: the classification of FNPs and their core biosynthetic enzymes, the evaluation and selection of diverse chassis strains, metabolic engineering strategies for heterologous NP production, and future perspectives in the discovery and heterologous expression of FNPs.

Fungal natural product types and skeleton enzymes

The classification of FNP structural types is determined by the backbone enzymes responsible for synthesizing their basic chemical cores. These enzymes include: polyketide synthases (PKSs), which produce compounds like yellow aflatoxins from acetyl-CoA/propionyl-CoA/malonyl-CoA, non-ribosomal peptide synthases (NRPSs), exemplified by penicillin, which is assembled through the condensation of amino acids (AAs) and other building blocks by multidomain NRPSs, and terpenoid synthases (TSs)/cyclases (TCs), such as those involved in gibberellin synthesis, utilizing isoprene units derived from acetyl-CoA (Figure 1). In the pursuit of new FNPs, chassis cells can be

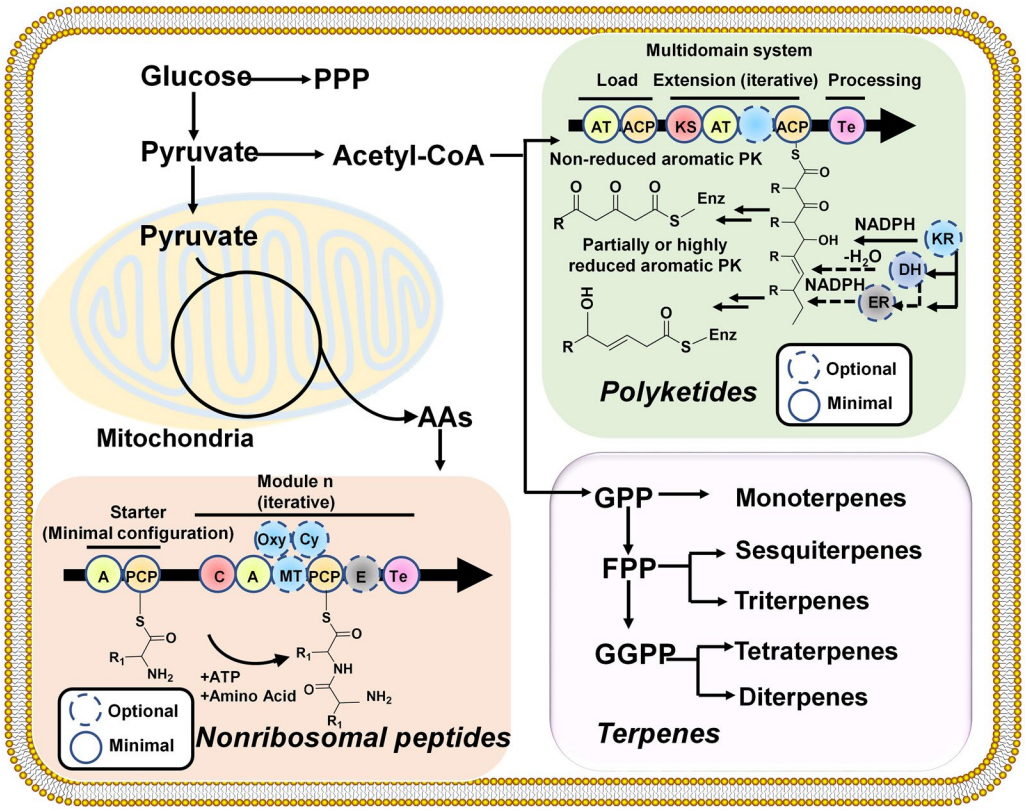

Figure 1. Brief overview of fungal secondary metabolite biosynthetic pathway. Three groups of secondary metabolites produced by fungi include polyketides, terpenoids, and non-ribosomal peptides. PPP: Pentose Phosphate Pathway. AAs: Amino Acids.

engineered to supply these precursors to different types of NPs.

Although strains may synthesize common polyketide precursors such as acetyl-CoA and malonyl-CoA endogenously, the formation of polyketides is frequently hindered by an insufficient supply of these precursors. Enhancing the availability of these precursors is a crucial metabolic engineering strategy to improve the titers of the end product [13]. Redirecting carbon flux toward the appropriate precursors has been the main focus of recent efforts [14]. For example, one acetyl-CoA and three malonyl-CoA molecules are utilized by *Penicillium patulum* to yield 6-methylsalicylic acid (6-MSA), an intermediate in the biosynthesis of the mycotoxin patulin [15]. Early research showed the feasibility of producing 6-MSA in yeast by co-expressing the 6-MSAS (polyketide synthase) gene and a PPTase [16]. Further studies have sought to increase 6-MSA production by augmenting the supply of the precursor malonyl-CoA. In one study, the native promoter of the *ACC1* gene, which is responsible for converting acetyl-CoA to malonyl-CoA, was replaced with a strong constitutive promoter (TEFp) in a strain that expressed the PPTase from *Aspergillus nidulans* and 6-MSAS from *Penicillium patulum*. This alteration led to a 60% increase in 6-MSA titer [17].

Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are the precursors of isoprenoids, differing in size, structure, and function. These two molecules combine to form geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) (Figure 1). GPP, FPP, and GGPP are then cyclized and/or rearranged by terpene synthases to yield monoterpenes, sesquiterpenes, and diterpenes, respectively (Figure 1). The diterpenoid pleuromutilin, synthesized by the mushroom *Clitopilus passeckerianus*, provides the starting point for the synthesis of the topical antibacterial antibiotic retapamulin [18]. An original patent application from Sandoz AG identified six transcriptionally co-regulated genes, including: a diterpene synthase gene, a putative geranylgeranyl diphosphate synthase gene, three cytochrome P450 monooxygenase genes, and a putative acyltransferase-encoding gene, outlining the pleuromutilin pathway [19,20]. The cDNA sequences of seven genes (*Pl-ggs*, *Pl-cyc*, *Pl-atf*, *Pl-sdr*, *Pl-p450-1*, *Pl-p450-2*, and *Pl-p450-3*) constitutively made up the gene cluster in *C. passeckerianus,* which was cloned and expressed with constitutive promoters in *A. oryzae* [21], resulting in a remarkable 20-fold increase in pleuromutilin titers compared with the native host. This accomplishment opens the door for future

improvements of pleuromutilin production in *A. oryzae* through pathway enzyme overexpression, marking the successful heterologous expression of an entire basidiomycete secondary metabolite gene cluster in an ascomycete chassis cell.

The diverse array of precursor monomers is fundamental to the formation of diverse structures and activities of non-ribosomal peptides (NRPs) [22,23]. However, this diversity poses significant challenges for heterologous production, as host organisms often lack the ability to synthesize most of these uncommon monomers. One approach to address this limitation involves cloning genes responsible for the monomer biosynthesis pathway [24]. Alternatively, precursor deficiencies can be overcome by directly supplementing the corresponding compounds to the culture medium [24]. Nonetheless, this strategy is not always successful, as negative feedback mechanisms may hinder compound production [24]. For instance, feeding the NRP precursor 2-aminobutyric acid to the culture medium of *C. asteris* was recently found to have a detrimental effect on astin C production and fungal development [25]. Moreover, the robust secondary metabolism of most filamentous fungi can compete for nutrients and precursors, potentially limiting heterologous NRP production. Modulation of metabolic fluxes and deletion of biosynthetic pathways involved in secondary metabolism can be favorable for the production of the target compound [26]. Additionally, reducing secondary metabolite production in the host can aid in detecting and purifying the desired compound by minimizing unwanted by-products [27]. The long-standing study of penicillin heterologous production exemplifies these principles. High-penicillin producing strains often harbor multiple copies of penicillin biosynthetic genes or have undergone deletions of secondary metabolite biosynthetic clusters observed in wild-type *Penicillium chrysogenum* NRRL195 [28]. Deleting these pathways has significantly improved strain productivity for β-lactam antibiotics [29].

Rational selection of ideal chassis

Chassis cells, which serve as hosts for heterologous expression of BGCs, provide the foundational components for the synthesis of NPs. These cells determine the biosynthetic substrate and metabolic flux, which in turn influence the nature and yield of NP synthesis. Therefore, selecting appropriate chassis cells based on the specific biosynthetic pathway and optimizing their metabolic processes are crucial for effective heterologous expression of FNPs.

Prokaryotic chassis

The prokaryotic expression system serves as a well-established host for heterologous expression due to its simple culture conditions, short growth cycle, and well-defined genetic background (Table 1). *Escherichia coli* is used primarily for expressing key enzymes in the biosynthetic pathways of FNPs [52]. For example, the PKS gene responsible for synthesizing 6-methylsalicylic acid in griseofulvin-producing bacteria has been successfully expressed in *E. coli* [16]. Additionally, *Bacillus subtilis* has emerged as another effective heterologous host for FNPs. Schweder et al. achieved successful expression of the non-ribosomal cyclic depsipeptide enniatin from *Fusarium oxysporum* in *B. subtilis* [34], marking the first expression of NRP production genes from eukaryotes in *B. subtilis*. Moreover, *B. subtilis* can excrete enniatin completely, which is advantageous for fermentation processes [34].

Prokaryotes also present limitations as heterologous hosts for reconstituting full pathways of FNPs. These constraints include challenges in efficiently expressing and correctly folding key fungal enzymes, often leading to the formation of inclusion bodies or difficulties in recognizing fungal promoters, terminators, and introns [53]. Protein expression can also be hindered by codon bias and the inability of tRNAs in prokaryotic hosts to recognize particular codons in foreign genes. Consideration must be given to a number of factors for successful heterologous expression in *E. coli*, including host strain selection and vector-specific considerations [54]. To obtain controlled expression that supports appropriate protein folding, variables such as gene copy number, promoter strength, and induction conditions could be appropriately adjusted at the translational level. In particular, the availability of: mRNA, tRNAs, ribosome binding site (RBS) secondary structure and amino acids [55], 5′ UTR sequences [56], and regulatory genes that regulate translation rates, all have an impact on translation efficiency. Moreover, fusion tags are frequently used to enhance mRNA stability, facilitate purification, and improve protein solubility. In 2024, researchers demonstrated the interplay between ribosomes and mRNAs that regulates the expression of recombinant proteins, providing a new perspective on the expression of heterologous substances in prokaryotes [57]. Further attempts to enhance expression include: cofactor regeneration, chaperone co-expression to reduce protein misfolding, optimization of protein secretion, and development of methods to circumvent feedback inhibition caused by product accumulation [58]. It is also important to note that different prokaryotes may face different problems. It has been demonstrated that when faced with the synthesis of the same substance, different

Table 1. Comparison of different heterologous hosts for production of fungal natural products.

Classification	Heterologous hosts	Advantages	Disadvantages	Heterologous products	Titers	References
Bacteria	*E. coli*	Fast growing; Simple for product analysis and isolation; Capacity for continuous fermentation	Inability to correctly splice introns; Lack of PPTase activity; Dependency on specific primary metabolites; Codon bias; Poor suitability for complex natural products production; Sensitivity to the metabolite being produced (*Streptomyces*); Instability of plasmids (*B. subtilis*)	6-dEB (PK); yersiniabactin (PK-NRP)	210 mg/L; 0.5-3 mg/L	[30,31]
	Streptomyces (*S. coelicolor, S. avermitilis S. tsukubaensis*)	Plenty of precursor supply; Availability of rapid screening methods; Most genetically studied actinomycete (*S. coelicolor*)		Hydropyrene and odyverdienes A (terpenes); tacrolimus (PK)	(--); 3746 mg/L	[32,33]
	Bacillus subtilis	Safe production profile; Well-characterized physiology; Various genetic manipulation tools		Enniatin (NRP)	1.1 mg/L	[34]
Yeast	*S. cerevisiae*	Rapid growth; Accessibility of scale-up in industry; Versatile genetic tools; No endotoxins production		Simvastatin; Penicillin G; FR901512; Bikaverin	55 mg/L; 5 ng/mL; (--); 202.75 mg/L	[35–38]
Filamentous fungi	*A. nidulans*	Ability to correctly splice introns; Possessing endogenous PPTase activity; Appropriate post-translational modifications; Genetically tractability; Ability to grow at a wide range of temperatures, pH values, water activity, and salinity (*Aspergillus* spp.); Various genetic tools, transformation methods and selection systems (*Aspergillus* spp.); GRAS status (*A. oryzae* and *A. niger*); Clean genetic background (*A. oryzae*); Mature fermentation technology, and species-specific traits, such as β-lactam specialization (*P. chrysogenum*)	Low relative growth rate; Complex metabolic network; Limited physiological knowledge; Unexpected side reactions by host enzymes (found in *A. oryzae*)	Asperfuranone;Geodin; Carotenoid; Citreoviridin; Neosartoricin B; Olivetolic acid	6.87 mg/L; 40–70 μg/plate; 125 μg/g dry mycelial; (--); 0.2 mg/L; 80 mg/L	[39–44]
	A. oryzae			Pleuromutilin; Tenellin; Erinacine Q;Aphidicolin	150 mg/L; 243 mg/L; 4.7 mg/L; 7.34 mg/L	[42,45,46]
	A. niger			Penicillin V; Beauvericin/ Bassianolide; Basidioferrin	2.3 mg/L; 350–600 mg/L; (--)	[47–49]
	Penicillium chrysogenum			Calbistrin; Pravastatin	(--); 6 g/L	[50,51]

(–): Titer not reported.

strains may have significant differences in expression, which creates a heterologous expression dilemma. For instance, there might be an 8-fold variation in yield between distinct strains of *Monascus pilosus* during the synthesis of monacolin K [59].

It is often essential to remove introns from the protein-coding regions and replace fungal promoters and terminators with host-specific elements to achieve heterologous expression of fungal genes in prokaryotic hosts. Additionally, optimizing the codons in the fungal sequence is typically necessary. These labor-intensive processes make a prokaryotic host less suited for expressing large fungal gene clusters as heterologous systems [54].

Yeast chassis

As a unicellular eukaryote, yeast is highly valued for its quick growth, simple culture conditions, and advanced gene editing tools (Table 1). As an effective expression system devoid of endotoxin, yeast excels in processing and post-translationally modifying fungal proteins within its secretory pathway, all while maintaining a clean genetic background [60]. Moreover, equipped with diverse synthetic biology tools, yeast, particularly *S. cerevisiae,* has proven to be instrumental in elucidating the biosynthetic routes of FNPs and developing new compounds. Versatile genetic manipulation tools have been developed for yeast species, such as: *S. cerevisiae*, *Pichia pastoris* and *Hansenula polymorpha*, facilitating BGC assembly and metabolic engineering. For example, the GTR-CRISPR system [61], utilizing a gRNA-tRNA array, was developed to enable multi-gene disruption and simultaneous integration at multiple loci [62]. In addition to gene expression through integration, it is also possible to express heterologous pathways in strains with various auxotrophic phenotypes by utilizing plasmids bearing the corresponding

auxotrophic markers [63]. Additionally, yeast species serve as excellent hosts with minimal endogenous secondary metabolites, providing clean backgrounds for metabolite purification and identification due to their low production of such compounds.

In existing studies, significant progress has been made in the functional expression of key biosynthetic enzymes in polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), with a focus on yeast as an expression system. It has been shown that integration of a phosphopantetheinyl transferase (PPTase) gene into the yeast genome is crucial for the functional expression of essential biosynthetic enzymes in PKSs and NRPSs [64]. The PPTase enzyme plays a vital role in catalyzing the attachment of the coenzyme 4′-phosphopantetheine moiety to PKS and NRPS, converting them from the dormant apo-form to the active holo-form. In various studies, *S. cerevisiae* BJ5464-NpgA has been developed as a host strain for expressing heterologous fungal BGC by overexpressing the *NpgA* gene that encodes the PPTase enzyme from *A. niger* [65]. Using dimethylbutyryl-S-methyl mercaptopropionate (DMB-SMMP) as the acyl-donor, expression of LovA and LovD in the engineered strain derived from *S. cerevisiae* BJ5464-NpgA produced 0.5 mg/L simvastatin. Simvastatin production was enhanced to 55 mg/L by utilizing an *in situ* cell lysis procedure and optimizing the expression level of the *lovA* gene [35]. Yeast expression systems have played a pivotal role in the synthesis of FNPs. For instance, Tang et al. [66] created a strong platform based on *S. cerevisiae* and added features, including: 30 auto-inducible promoters, better mitochondrial stability, increased sporulation of *S. cerevisiae*, and overexpression of the *npgA* gene from *A. nidulans* and P450 reductase from *Aspergillus terreus*. With the use of this platform, 41 BGCs from fungi were successfully expressed heterologously, and more than half of them produced measurable amounts of different chemicals (Figure 2(a)).

While yeast is indispensable for FNP biosynthesis and serves as a key organism in synthetic biology research, it does have its limitations. The absence of endogenous secondary metabolism in yeast implies that it is not inherently ideal for FNP synthesis. Furthermore, the absence of advanced mRNA splicing machinery in yeast necessitates the excision of introns for gene expression. To address these challenges, there is a pressing need for the development of more sophisticated and engineered yeast strains to function as effective heterologous hosts. This underscores the ongoing necessity for significant advancements in this field.

Filamentous fungal chassis

Compared to the prokaryotic and yeast heterologous hosts mentioned above, filamentous fungi offer a significant advantage: most filamentous fungal hosts are capable of accurately processing intron splicing from other fungal secondary metabolite genes, thereby facilitating successful production of the target product (Table 1) [69–71]. Additionally, filamentous fungi naturally possess endogenous PPTases, eliminating the need for engineering post-translational modifications of PKSs and NRPSs. Currently, numerous fungi have been successfully utilized in biosynthetic pathway research and the heterologous expression of FNPs, including *Aspergillus nidulans*, *Aspergillus oryzae*, and *Aspergillus niger* (Table 1).

A. nidulans, one of the earliest sequenced model fungi, possesses robust capabilities for secondary metabolite production and advanced molecular genetic tools, including: easy molecular manipulation, mature genetic transformation technologies, and an efficient gene targeting system [2]. The fungus undergoes both sexual and asexual reproduction stages, facilitating the creation of multi-gene mutant strains and supporting studies on secondary metabolite biosynthesis in other fungi [69]. To enhance *A. nidulans* as a host for heterologous expression, collaboration among three research groups led to the development of efficient methods for rapid knockout of secondary metabolite genes and entire gene clusters [39]. Various mutants were constructed to simplify the strain background by deleting native metabolites like sterigmatocystin and emericellamide, minimizing interference with the detection and isolation of heterologous FNPs [72]. The *A. nidulans* expression system has successfully produced numerous FNPs. For instance, Ryan et al. expressed a segment of the ergot alkaloid precursor chanoclavine-I gene cluster from *A. fumigatus* in *A. nidulans*, achieving production of chanoclavine-I and gaining insights into the early ergot alkaloid biosynthesis [73]. They also successfully expressed the complete gene cluster of geodin from *A. fumigatus* in *A. nidulans*, demonstrating the system's capability to discover novel secondary metabolites and construct artificial biosynthetic pathways [40]. A recent development is the co-inducible nitrate (CoIN) expression system for NPs based on *A. nidulans* (Figure 2(b)) [41]. This system leverages the transcription factor (AflR) and its cofactor (AflS) of the sterigmatocystin (ST) BGC, which can effectively regulate other genes within the ST BGC. The *aflR* and *aflS* promoters were substituted with a bidirectional nitrate-inducible promoter (*niaD*/*niiA*), which can regulate the expression of the promoter regions within the ST BGC. As a

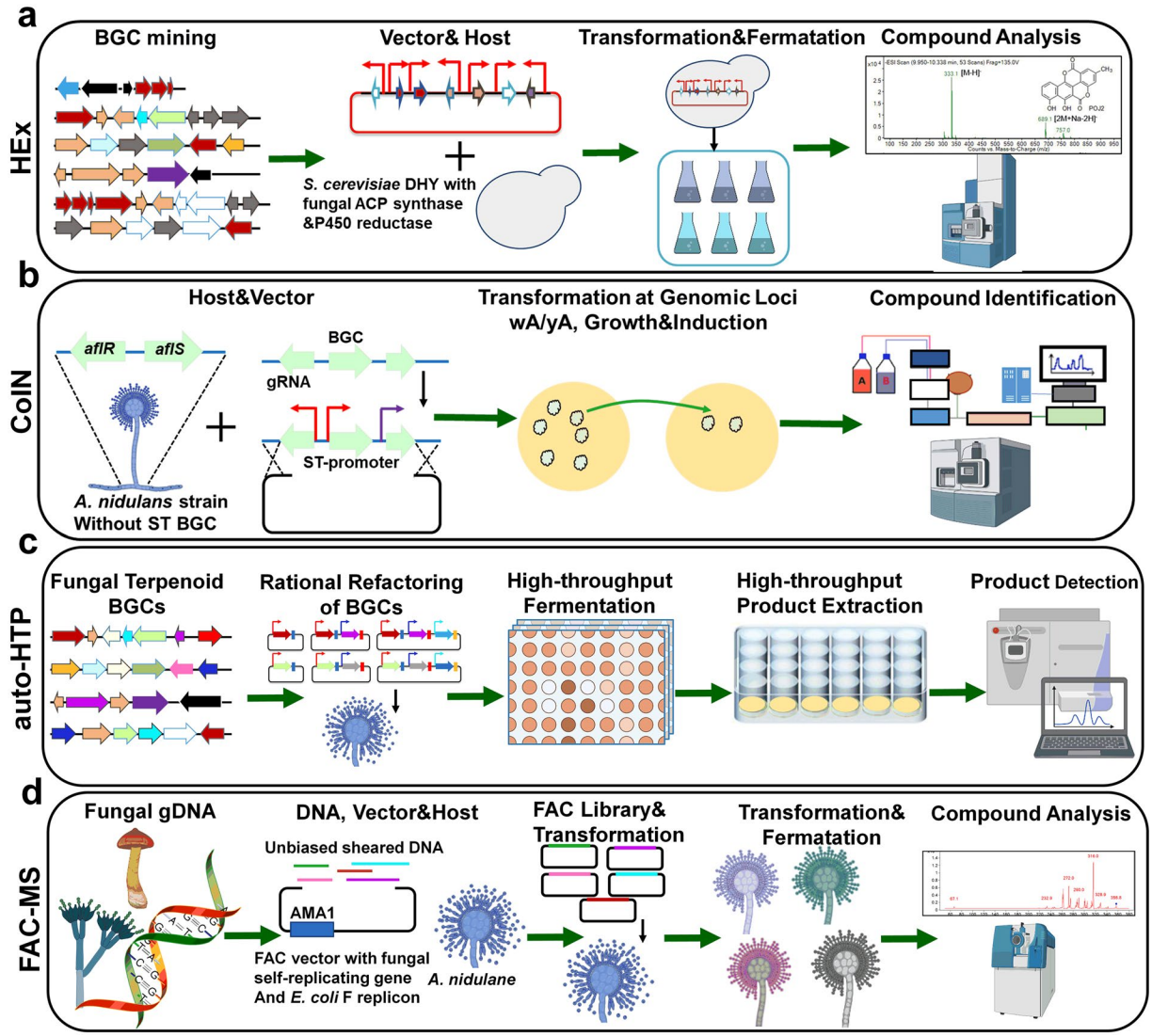

Figure 2. Schematic of four heterologous platforms recently developed to characterize novel fungal secondary metabolites. a. A platform called heterologous expression (HEx) was recently developed in *S. cerevisiae* [66]. b. The co-inducible nitrate (CoIN) expression system for NPs in *A. nidulans* is designed to study of BGCs with unknown products, especially when these products may be toxic to the host [41]. c. An *A. oryzae*-based platform efficiently provides precursors for fungal terpenoids [67]. d. A novel heterologous platform using *A. nidulans* with a self-replicating fungal artificial chromosome (FAC) system [68].

proof of concept, the three genes of β-carotene biosynthesis were controlled by specific ST promoters, resulting in a high-yield production of β-carotene upon induction. This CoIN system shows promise for exploring unknown BGCs, particularly for FNPs that may be toxic to the host (Figure 2(b)).

A. oryzae, known for its role in traditional soy sauce and alcohol fermentation, is also highly regarded as a safe and efficient industrial production strain [69]. This filamentous fungus secretes metabolites prolifically into the medium, benefiting from well-established fermentation technologies that support scale-up to industrial production levels. Moreover, *A. oryzae* possesses a clear genomic background and capabilities for splicing introns and post-translational modifications [74]. These characteristics make *A. oryzae* an excellent natural heterologous host of fungi, providing benefits for both rapid industrial production and laboratory studies. *A. oryzae* has been successfully used for heterologous production of FNPs. For instance, the compound naphthopyrone was obtained by heterologous expression of the *alb1* gene from *A. fumigatus* in *A. oryzae*, and the function of Alb1p PKS was confirmed [75]. Similarly, various compounds, such as: tenellin, aphidicolin, paxilline, and aspyridone from different fungi, have been synthesized and expressed *de novo* in *A. oryzae* [76]. In another case, pleuromutilin production increased 10-fold to 84 mg/L when seven pleuromutilin biosynthesis genes were heterologously expressed in *A. oryzae* utilizing constitutive promoters [21]. This illustration

shows how effectively BGC expression in *A. oryzae* can produce NPs, aiding in the discovery of novel chemicals. It can be seen that the reconstruction of the biosynthetic mechanism in the heterologous host *A. oryzae* allows the elucidation of each enzymatic reaction step in the synthesis process, as well as the identification of intermediates and final NPs. Interestingly, a platform based on *A. oryzae* efficiently provides precursors for fungus-derived terpenoids (Figure 2(c)). In the *A. oryzae* microbial chassis, a high-throughput automated bio-foundry platform is utilized for rapid and rational reconstruction [77] and combinatorial rearrangement of synthetic genes, overcoming challenges, such as: low-throughput, low-yield, high-repetition rate, and unknown biosynthetic mechanism typical in NP discovery. Moreover, the integration of techniques like AlphaFold2 structure prediction has enabled the excavation and screening of numerous terpenoids with novel structures, potent activity, and efficient synthesis within this chassis [78]. This streamlined approach not only advances the study of FNPs but also solidifies *A.oryzae* as a robust platform for studying FNPs and their synthetic pathways.

A. niger is another important strain widely used in industry and is certified as a safe strain. In 1990, the FNP penicillin was first heterologously expressed in filamentous fungi, with *A. niger* being one of the selected hosts [47]. When it comes to producing NRPs, *A. niger* is a highly advantageous heterologous host, especially in industrial settings. This fungus is amenable to genetic manipulation, benefiting from advanced tools and techniques that enable the introduction of heterologous genes and the optimization of metabolic pathways to enhance NRP production. With a robust secretion system, *A. niger* also facilitates the efficient secretion of NRPs into the culture medium, streamlining downstream processing and purification. Additionally, this fungus can be cultivated to yield high NRP titers, thriving in simple, cost-effective media and generating substantial biomass for industrial-scale production. For example, the NRPS gene *esyn1* from *Fusarium oxysporum* was expressed by Cairns et al. using the Tet-on method, resulting in the production of approximately 5 g/L enniatin [79]. Furthermore, the fungal cyclodepsipeptides beauvericin and bassianolide, which are derived from *Beauveria bassiana,* were produced at high titers (350–600 mg/L) by overexpressing their corresponding NRPS in *A. niger* [48]. To date, an increasing number of FNPs can be produced using various heterologous expression platforms (Table 1), showcasing the power of these methods in discovering new compounds, including those from unculturable strains, producing drug precursors or drugs, and exploring NP biosynthetic pathways (Figure 2(d)). However, the specific characteristics of BGCs, such as unique precursors and toxicity, as well as the choice of expression vectors can affect the production levels reached by heterologous expression. Thus, additional strategies are required to maximize output.

Reconstituting biosynthetic pathways

FNP BGCs typically comprise core enzymes, tailoring enzymes, transporters, and self-resistance proteins, totaling around 10 genes [80]. A significant obstacle in the process of expressing a fungal BGC heterologously is assembling and transferring the entire BGC into a non-native host. Due to the complexities involved in genetic modifications, this process has proven to be difficult.

Various methods have been developed to address the cloning and expression of fungal BGCs in filamentous fungal hosts. It has been found that heterologous expression of BGCs often requires the replacement of the original promoter with a host-compatible promoter, with different promoter strengths influencing gene expression. Therefore, one strategy involves independently cloning each gene along with its promoter and terminator sequences within the BGC and subsequently assembling these genes. These constructed components are then gradually integrated into the chassis strain's genome [81]. By replacing the original promoter with a new one, formerly silenced genes can be properly expressed in either the original or heterologous host. This approach facilitates the combinatorial expression of biosynthetic genes, which is widely utilized for characterizing BGCs in the context of FNPs and conducting functional analyses of key enzymes. For example, considering that fungal secondary metabolites may be toxic to the host, researchers often opt for an inducible promoter to activate previously silenced genes, enabling the generation of new active FNPs without compromising fungal growth. Six genes from the *Aspergillus nidulans* genome were discovered to be part of a silenced gene cluster by Yeh and colleagues. The authors simultaneously substituted the alcA promoter for the promoters of these six genes in order to investigate the function of this gene cluster. They eventually discovered that this silenced gene cluster is responsible for the production of the protease inhibitor fallutamide B [82].

Fungal BGCs are complex, multigene structures that typically span hundreds of kilobases in size. Direct cloning, a method involving the construction of a library of cosmids or fungal artificial chromosomes (FACs) to transfer the whole BGC into a heterologous

host in a single step, has been used to study fungal BGCs. Large gene fragment cloning was enabled by the FAC method, introduced by the Keller laboratory in 2015 [68]. This technology can transport DNA fragments up to 150 kb with an AMA1 sequence that supports autonomous replication in filamentous fungi. This technology facilitates the rapid extraction of genes of varying sizes from the natural host's genomic DNA. Using this technique, 56 *Aspergillus terreus* gene clusters have been identified thus far. Terezine D, an intermediate in *A. terreus*'s astechrome synthesis pathway, has been successfully identified in the heterologous host *A. nidulans*, serving as a proof-of-concept. The development of FACs-based direct cloning provides a useful tool for the investigation and heterologous synthesis of fungal BGCs. However, this method requires artificial gene splicing.

When applied in yeast, the yeast transformation-associated recombination (TAR) method has proven to be reliable and effective for assembling numerous genes from genomic DNA into large intact fungal BGCs [83,84]. Large BGCs are cloned into multiple DNA fragments using this approach, with homologous arms inserted between each fragment and the vectors, which are co-transformed into *S. cerevisiae* to facilitate homologous recombination assembly. Li et al. evaluated this approach by assembling 14 fungal BGCs with sizes ranging from 7 kb to 52 kb, demonstrating high efficiency with an average positive rate of more than 80% [83]. In another study, the 9.9 kb cytokinin BGC (FCK) and the 54 kb W493 BGC (a PKS-NRPS hybrid gene cluster) of *Fusarium pseudograminearum* were amplified by PCR in 3 and 16 fragments, respectively, with 50 bp homology arms between adjacent fragments [84]. The purified PCR fragments were efficiently assembled in yeast. However, it is important to note that while effective, this method is laborious and time-consuming.

With the advent of clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) tools [85], the assembly of multiple fragments constituting entire BGCs has been accomplished in a single step. Inhibiting non-homologous end joining DNA repair by deleting Ku70, Ku80 or Lig4 was used to increase the efficiency of homologous recombination [50]. For example, the calbistrin BGC from *P. decumbens* was amplified as six PCR fragments, each with neighboring homologous arms spanning from 100 bp to over 2000 bp, which were simultaneously transformed into a *P. rubens* strain lacking Ku70 using the CRISPR-Cas9 method [50]. The proper assembly efficiency of calbistrin BGCs in the selected transformants reached 100%, indicating that the CRISPR-Cas9 approach works well for quickly creating heterologous BGCs with high homologous recombination efficiency [50]. Additionally, a recyclable CRISPR-Cas9 genome editing system was established in *A. oryzae* to speed up the assembly of natural product biosynthesis pathways [86,87]. Using just two rounds of transformation, the recurrent multiplex genome editing approach was able to effectively assemble all 10 erinacine biosynthetic genes into *A. oryzae* [88], proving its usefulness for quickly reconstituting heterologous BGCs. The advancements in CRISPR-Cas9 technology have paved the way for engineering and analyzing natural product pathways through the construction and modification of BGCs.

Recently, efforts have been made to explore more efficient and alternative approaches in the development of gene expression systems. For example, a polycistronic form containing 2 A peptides has been used to heterologously express several genes within a single BGC under a single promoter [89]. The 2 A peptide acts as a signal to regulate translation between enzymes, ensuring equimolar expression of the enzymes. In order to aid in the selection of appropriate transformants and enable the assembly of the polycistron of numerous genes within a BGC, a user-friendly vector containing 2 A peptides and a split fluorescent reporter protein was developed [90]. The BGC from the mushroom *Psilocybe cubensis*, consisting of four genes, was assembled as a polycistron under the control of a Tet-on promoter and transformed into *A. nidulans*, leading to the production of psilocybin with a titer of 110 mg/L [90]. However, limitations exist, such as the limited number of genes that can be cloned and variations in gene expression levels based on their position within the polycistron [90,91].

Overall, the development of large fragment gene cloning technology offers an essential technical basis for the heterologous synthesis of FNPs and the cloning of gene clusters. This will greatly accelerate research on the heterologous expression and production of FNPs.

Compartmentalized biosynthesis of fungus-natural products

Eukaryotes possess organelles that are delineated by single or double membranes [92]. Each organelle provides a unique physicochemical environment, including variations in pH and redox potential, as well as unique enzymes and metabolites [93]. By compartmentalizing biosynthetic pathways into distinct organelles, enzymes can gain better access to specific substrates, thereby improving pathway efficiency and reducing by-product synthesis (Figure 3). Compartmentalization can also increase the local

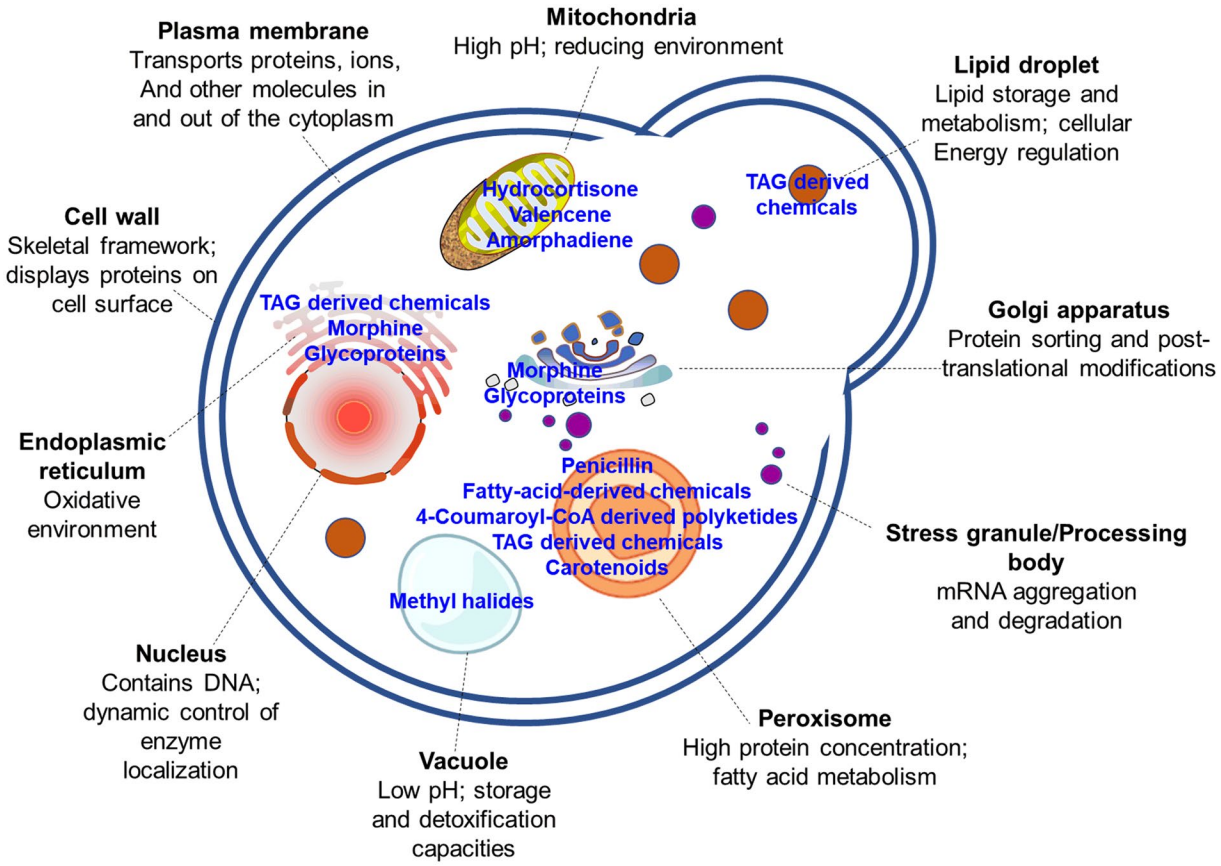

Figure 3. Overview of yeast subcellular compartments and compartmentalization engineering in different organelles. Eukaryotic cells contain various subcellular organelles with unique microenvironments, each providing favorable conditions for different metabolic pathways.

concentration of substrates and coenzymes, promoting the progression of the reaction.

The division of FNP biosynthetic pathways has been reported to occur naturally in filamentous fungi [94,95], such as in the biosynthesis of penicillin [96] and mycophenolic acid [96]. When it comes to penicillin production, an NRPS synthesizes a tripeptide using precursor amino acids from the vacuole. The vacuolar membrane-associated transporter PenV has been identified to facilitate penicillin G biosynthesis by transporting L-cysteine and L-valine from the vacuole to the cytoplasm, where the δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase is located [97]. After the ACV tripeptide is converted, isopenicillin is transported to the peroxisome and converted to penicillin G by a peroxisome-localized acyltransferase, which catalyzes the exchange of the α-aminoadipyl side chain with CoA-activated phenylacetic acid. It has also been reported that the phenylacetyl-CoA ligase enzyme, catalyzing the transfer of CoA to phenylacetic acid, has also been reported to localize in the peroxisome [96]. Furthermore, the uptake of phenylacetic acid and isopenicillin into peroxisomes has been revealed to be attributed to two peroxisome membrane-associated transporter proteins, PaaT and PenM [98]. As a result, studies have demonstrated the necessity of peroxisomes for penicillin G synthesis, even in a heterologous host like baker's yeast [36]. Du and Li have thoroughly investigated additional instances of fungal secondary metabolite synthesis in several organelles [96]. According to recent research, FNP production can be efficiently increased by targeting heterologous biosynthetic pathways to particular organelles [93].

Peroxisomes and mitochondria are the two organelles that are most frequently used to segregate biosynthetic pathways [99]. The mitochondria of yeast have been used in a variety of NP syntheses, including the synthesis of plant terpenoids, since they include a variety of metabolites and cofactors [100]. Further investigation is necessary to determine whether filamentous fungal mitochondria are equally appropriate for producing heterologous NPs. Peroxisomes, which are single-membrane-bound organelles found in the cytoplasm, are usually involved in the breakdown of hydrogen peroxide, the generation of acetyl-CoA

through the β-oxidation of fatty acids, and other metabolic processes [101]. It is well known that peroxisome size and proliferation can be precisely regulated to optimize functionality [101]. Several investigations have demonstrated the synthesis of various compounds in peroxisomes, such as squalene, monoterpenoids, plant alkaloids, and derivatives of the triacylglycerol pathway, showcasing their potential for optimizing precursor availability and sequestering toxic substances [102]. Additionally, peroxisomes function as organelles for storage, sequestering harmful substances that are created. For example, overexpression of the (R)-(+)-limonene synthase *Cl*LimS and all the enzymes involved in the mevalonic acid (MVA) pathway in the cytosol produced 1.13 mg/L of (R)-(+)-limonene during the production process [103]. Targeting the mutant GPP synthase Erg20p and *Cl*LimS to peroxisomes, on the other hand, resulted in a 32-fold increase in (R)-(+)-limonene synthesis, reaching 35.68 mg/L. Further research is needed to explore the full potential of peroxisomes and mitochondria in the synthesis of heterologous natural products.

Prospectives

The emergence of extensive fungal genome sequencing data and its analysis has revealed that the number of BGCs in fungi far exceeds the number of discovered FNPs. Despite the vast potential of FNPs [104], many BGCs in fungi remain silent or minimally expressed under laboratory culture conditions. Moreover, the majority of fungi found in nature are unculturable, which makes it difficult to separate and identify the bioactive metabolites from these species. Heterologous expression platforms offer a promising method for activating fungal-derived BGCs. However, significant challenges persist in this approach. For example, current bioinformatics methods struggle to differentiate between truly inactive gene clusters and those that are dormant due to environmental cues. In the future, it is necessary to introduce artificial intelligence and big data analysis methods to improve the identification of silent and inactivated gene clusters. Furthermore, the development of gene repair technologies for inactive genes can enhance the activation efficiency of silent gene clusters (Figure 4(a)).

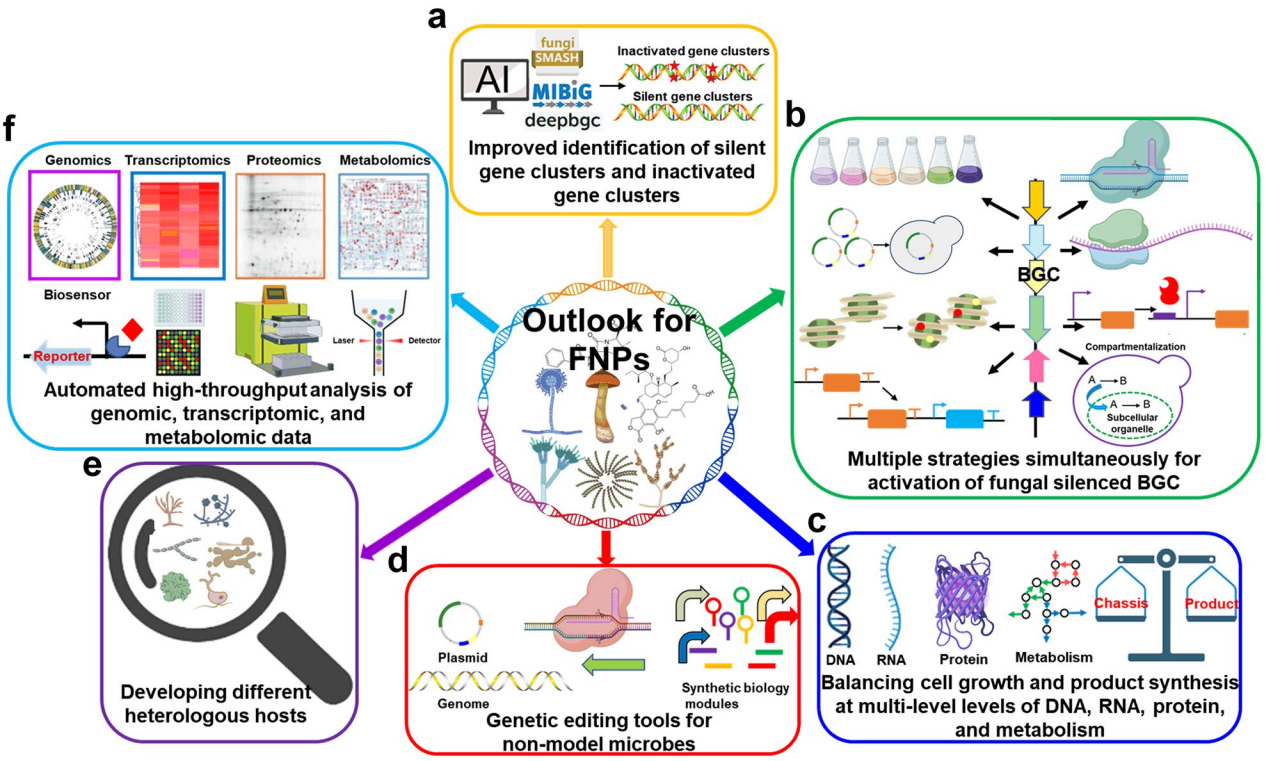

Figure 4. Future perspectives on the construction of chassis cells for FNP production. a. In the future, silent gene clusters and inactivated gene clusters can be identified efficiently by artificial intelligence and big data analysis method, thereby facilitating the discovery of FNPs. b. Multiple activation strategies for fungal silenced BGCs should be utilized simultaneously to accelerate the discovery process of new FNPs. c. Efficient, controllable, and stable expression of FNP biosynthetic pathways in chassis cells will be realized by balancing cell growth and product synthesis at multiple levels (DNA, RNA, protein, and metabolism). d. Genetic editing tools for non-model microbes should be developed to expand the range of BGC expression hosts. e. Diverse heterologous hosts need to be developed in the future to accommodate specific sources. f. Automated high-throughput analysis of genomic, transcriptomic, and metabolomic data, enabling more efficient gene-compound mapping, will be realized by combining genomics, transcriptomics, proteomics, metabolomics, high-throughput screening biosensor, and automated high-throughput instruments.

Various strategies have been devised to activate silenced gene clusters, resulting in the discovery of numerous natural compounds with novel structures and remarkable activities [88]. However, each strategy comes with its own advantages and limitations, making it challenging to fully activate silenced gene clusters in fungi using a single approach. Therefore, it is crucial to adopt multiple strategies simultaneously to maximize the activation of silenced gene clusters (Figure 4(b)).

The synthesis pathways of FNPs are intricate, involving multi-step enzyme-catalyzed reactions. Expressing these pathways in heterologous hosts presents challenges not only in assembly but also in adaptation. Balancing the relationship between the heterologous synthesis pathway and the growth and metabolism of the chassis cells is a critical challenge in heterologous expression. Future research should focus on understanding the adaptability principles at different levels (DNA, RNA, protein, and metabolism) to achieve rapid assembly and efficient, controllable, and stable expression of FNP synthesis pathways in host cells (Figure 4(c)).

Advancements in gene editing and metabolic regulation tools offer opportunities for intelligent regulation of microbial metabolic flow to balance cell growth and product synthesis in building smart cell factories. These include developing real-time monitoring and high-throughput screening technologies, understanding the transport mechanism of key metabolites, designing efficient orthogonal genome editing and transcriptional resource allocation technologies, and enhancing the efficiency of heterologous expression of FNPs (Figure 4(c)). However, the lack of efficient genetic manipulation poses a major obstacle to the activation of silent gene clusters in many non-model strains. For instance, introducing foreign genes may be difficult, and there may insufficient screening techniques for the selection of transformed strains. To overcome these obstacles, more work is needed to develop genetic transformation strategies and screening techniques for non-model fungi (Figure 4(d)).

While a variety of hosts have been used to synthesize fungal-derived natural products, such as yeast and filamentous fungal strains, expanding the repertoire of heterologous hosts is crucial for exploring NPs from diverse fungal sources, particularly Basidiomycetes. Evolutionarily related hosts offer advantages such as compatible intron splicing machinery and codon usage bias. By removing natural BGCs and modifying metabolic pathways related to precursor biosynthesis, these hosts can be further tuned to provide flexible platform strains. To effectively produce specific FNPs, a range of fungal heterologous hosts that are suitable for the products and their precursors must be investigated. Currently, the number of heterologous hosts capable of expressing fungal silenced gene clusters is still limited, necessitating the development of additional heterologous hosts to accommodate specific sources (Figure 4(e)).

Drug compounds can be derived from FNPs and their derivatives. Nevertheless, issues like reproducibility and the complex nature of metabolites pose difficulties for conventional techniques to obtain FNPs. In addition to being time- and labor-intensive, these techniques also have difficulty in unlocking the "black box" of metabolite synthesis. With the rapid development of genome sequencing technology, genomics-guided FNP discovery has become essential in the pharmaceutical research field. Rahim et al. assembled the genomes of 20 different strains through seven sequencing experiments, linking BGCs to N,N-dimethylcycloditryptophan, two novel lasso peptides, and three metabolites known to be actinomycetes-associated iron carriers [105]. The integration of multi-omics technologies, systems biology approaches, and theories is driving the automated high-throughput analysis of genomic, transcriptomic, and metabolomic data. This enables more efficient mapping of genes to compounds (Figure 4(f)). Based on the combined use of these theories and the update of network tools, many novel mining techniques have been developed. These techniques prioritize data integration from chemical structures, genomes, and metabolomics. The reform and innovation of FNP research is creating a multi-field and multi-disciplinary research model, integrating diverse learning methods, theoretical foundations and real-time network informatics technology. Researchers have also started using AI in related production investigations in recent years [106]. Looking ahead, the maturation of artificial intelligence technology, coupled with decreasing costs of genome sequencing, enhanced efficiency of high-throughput gene editing, and further breakthroughs in high-throughput cloning of BGCs, will accelerate the development of new drugs derived from fungi. These advancements hold the promise of revolutionizing drug discovery by expanding our ability to explore and harness fungal-derived natural products for therapeutic applications.

The development of heterologous platforms using synthetic biology approaches will greatly facilitate the rapid exploration of FNPs by unlocking the vast array of cryptic BGCs from fungi [88]. These developed heterologous platforms also offer the potential for producing novel bioactive compounds that may not exist in nature. This can be achieved through the

combinatorial expression of biosynthetic genes from different organisms or through engineering biosynthetic enzymes on scaffolds.

Author contributions

Junyang Wang, Zihe Liu and Shuobo Shi outlined this manuscript. Junyang Wang drafted the manuscript. Shuobo Shi, Zihe Liu and Xu Ji revised the manuscript. Zhenlin Xin, Xu Ji and Jinmiao Hu summarized the literature. All authors contributed to the article and approved the submitted version.

Disclosure statement

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ORCID

Shuobo Shi http://orcid.org/0000-0002-0261-3105

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