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Prospects for cordycepin biosynthesis in microbial cell factories

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Cordycepin, an adenosine analog, exhibits diverse bioactivities and holds significant potential for applications in healthcare and agriculture. Fungi of the genus *Cordyceps*, such as *Cordyceps militaris*, can naturally produce cordycepin. Current sources of cordycepin primarily involve extraction from fruiting bodies or isolation from liquid fermentation using *C. militaris*, presenting challenges such as low production intensity, complex separation and purification systems, and high production costs, limiting industrial feasibility. Recent advancements have witnessed the utilization of various fungal chassis cells to successfully engineer heterologous biosynthetic platforms for cordycepin, such as *Saccharomyces cerevisiae* and unconventional yeasts, offering advantages of high yield, short fermentation cycles, and a broad substrate spectrum. This mini review summarizes the biosynthetic pathways of cordycepin and focused on the comparison of the characteristics, advantages, current performance and prospects for the microbial cell factories, analyzing potential targets for metabolic pathway modification and giving strategies in both genetic engineering and process engineering to enhance production intensity. The mini review particularly emphasizes the crucial role of chassis cell stress tolerance to the toxic product in determining cordycepin yield and highlights the urgent need for high-throughput screening methods for high-yield strains.

KEYWORDS

cordycepin, *Cordyceps militaris*, biosynthesis, non-conventional yeast, microbial cell factory

1 Introduction

Cordycepin (3′-deoxyadenosine) is a unique adenosine analog first identified by Cunningham et al. (1950) in the fermentation broth of the medicinal fungus *Cordyceps militaris*. As a valuable natural compound, cordycepin exhibits a range of biological functions (Tuli et al., 2014; Radhi et al., 2021), including antitumor (Nakamura et al., 2015; Khan and Tania, 2023), anti-inflammatory (Tan et al., 2020), antiviral (Rabie, 2022), immunomodulatory activities (Lee et al., 2020). It also helps alleviate symptoms of diabetes (Shin et al., 2009) and hyperlipidemia (Guo et al., 2010), showing broad prospects for drug development and health products (Kunhorm et al., 2019; Yang et al., 2020). To relieve the rapid deamination of cordycepin *in vivo*, cordycepin derivatives with N_6 protection (Schwenzer et al., 2021; Cui et al., 2024) have been developed, and the ProTide NUC-

TABLE 1 Summary of cordycepin production in microbial cell factories and *C. militaris*.

Genetic background	Genetic modification	Cordycepin yield & productivity	Fermentation strategy	Deamination of cordycepin
S. cerevisiae BY4741 (Wang et al., 2024)	*Δado1*; *ura3::PGK1p- EA-ADH1t-TEF1p-EA2-CYC1t*; *HO:: pTHD3-ADE4 -tADH1*; *HO:: pTHD3-cpdBN-tADH*	725.16 mg/L & 5.03 mg/(L·h)	Fed-batch fermentation with pH optimization at 5.5	Not observed
S. cerevisiae S288c (Huo et al., 2021)	Episomal vector pYES2-Kan, *TDH3p-ScCNS1-CYC1t*, *TEF1p-ScCNS2-ADH1t*	137.27 mg/L & 0.95 mg/(L·h)	Fed-batch fermentation supplemented with 5 mmol/L Cu^{2+} and 1.0 g/L adenine	Not observed
Komagataella phaffii GS115 (Tan et al., 2023)	*AOX1::AOX1p-CNS1- RPS25At -FLD1p-CNS2-AOX1t*	2.68 ± 0.04 g/L &15.95 mg/(L·h)	Flask fermentation with optimization of adenine/methanol concentration	Yes, under weakly alkaline pH at 7.0 or 8.0
Yarrowia lipolytica YlCor-18 (Song et al., 2023)	Po1f-Δku70, *rDNA::up-TEFp-CmCns1-XPR2t-TEFp CmCns2 LIP2t-URA3-rDNA down*, *rDNA::up-TEFp-NK-XPR2t-TEFp PGK-LIP2t-LEU2-rDNA down*	4,362.54 mg/L (213.85 mg/g) & 26.0 mg/L/h	Fed-batch fermentation supplemented with 1.0 g/L of adenine	Not observed
Yarrowia lipolytica YLC22 (Duan et al., 2022)	Po1f-ΔKu70, *URA3*, *rDNA::FBAp-Cns2-XPR2t*, *TEFp-Cns1-LIP2t*, *LEU2*, *rDNA::TEFp-Cns3NK-2A-ADE4-2A-ADE12-LIP2t*	3,249.58 mg/L (3,588.59 mg/L from glucose)	A mixture of molasses and glucose as substrate in flask fermentation	Not observed
Yarrowia lipolytica YL-D05 (Duan et al., 2023a)	Po1f-ΔKu70, *URA3*, *rDNA::FBAp-Cns2-XPR2t*, *TEFp-Cns1-LIP2t*, *LEU2*, *rDNA::TEFp-GLK1-2A-PGM1-LIP2t*	2,286.04 mg/L (120 h) & 27.34 mg/L/h (72 h)	Flask fermentation with agro-industrial residues as substrate plus optimization of carbon source, nitrogen source, C/N ratio, and initial pH	Not observed
Aspergillus oryzae (Jeennor et al., 2023)	*PyrG::AnPgpdA-Cns2-AnTrpC-AoPgpdA-Cns1-AoTrpC*	1,129.29 ± 19.17 mg/L & 564.64 ± 9.59 mg/L/d	Submerged fermentation supplemented with adenine	Yes
C. militaris mutant G81-3 (Masuda et al., 2011)	The mutant obtained by high-energy proton beam irradiation	8,600 mg/L & 11.94 mg/L/h	Surface liquid culture supplemented with adenosine	Not observed
C. militaris mutant *GYS60* (Zhang et al., 2020)	The mutant obtained by multifunctional plasma mutation system	7,882.70 mg/L & 21.90 mg/L/h	Submerged liquid culture	Not observed
Recombinant *C. militaris* CM-adss-5 (Zhang et al., 2023)	Over-expressing genes encoding adenylosuccinate synthase, adenylosuccinate lyase, and 5′-nucleotidase genes	2,581.96 ± 21.07 mg/L & 8.97 mg/L/h	Submerged liquid culture under optimized culture conditions	Not observed

7738, featuring a protective phosphoramidate cap, has overcoming cellular resistance (Schwenzer et al., 2021) and is currently in Phase II clinical trials. Cordycepin directly inhibits cancer cell proliferation and disrupts the tumor microenvironment (Khan and Tania, 2020; Chen et al., 2024), making it suitable for combination therapies (Liao et al., 2020). Additionally, cordycepin exhibits antibacterial (Jiang et al., 2019), insecticidal (Woolley et al., 2020), and allelopathic effects (Quy et al., 2019) comparable to glyphosate in dicotyledonous plants, indicating potential agricultural applications. However, research on the mechanisms underlying cordycepin's activity has predominantly focused on mammalian cells, involving pathways such as PI3K/Akt/mTOR, MAPK and AMPK signaling (Radhi et al., 2021), with limited data on microbial or plant systems. Phosphorylation of cordycepin (CoTP) (Hawley et al., 2020) plays a central role in its activity.

Cordycepin can be obtained via chemical synthesis and biological methods (Wang et al., 2022). Chemical synthesis faces challenges of efficiency, cost, precursor availability, and environmental concerns (Hansske and Robins, 1985; Aman et al., 2000), making it unsuitable for industrial applications. Biological methods mainly involve using naturally cordycepin-synthesizing microorganisms or artificially constructed microbial cell factories through solid or liquid fermentation (Duan X. et al., 2023). Fungi with natural cordycepin biosynthesis pathways include *C. militaris* (Xia et al., 2017), *Ophiocordyceps sinensis* (Xiang et al., 2014), *Cordyceps kyushuensis* (Zhao et al., 2019), *Cordyceps cicadae* (Liu et al., 2018), *Aspergillus nidulans* (Xia et al., 2017), and *Irpex lacteus* (Liu et al., 2022). These fungi employ various fermentation techniques, such as solid-state fermentation, static fermentation, and submerged fermentation. Previous review articles have analyzed the evolutionary relationships among these fungi and compared their yields (Wang et al., 2022; Duan X. et al., 2023). Currently, extraction from *C. militaris* fruiting bodies or fermentation broth remains the primary source of cordycepin.

Although *C. militaris* exhibits the highest fermentation yield among known natural cordycepin hosts (Duan X. et al., 2023), its long fermentation cycle results in low production intensity. Solid and surface fermentation typically require 45–60 days, and submerged liquid fermentation takes at least 15–20 days (Yang et al., 2020). Additionally, the strains are highly unstable, facing issues such as strain degeneration (e.g., abnormal color change, failure to form fruiting bodies) (Lou et al., 2019) during fruiting

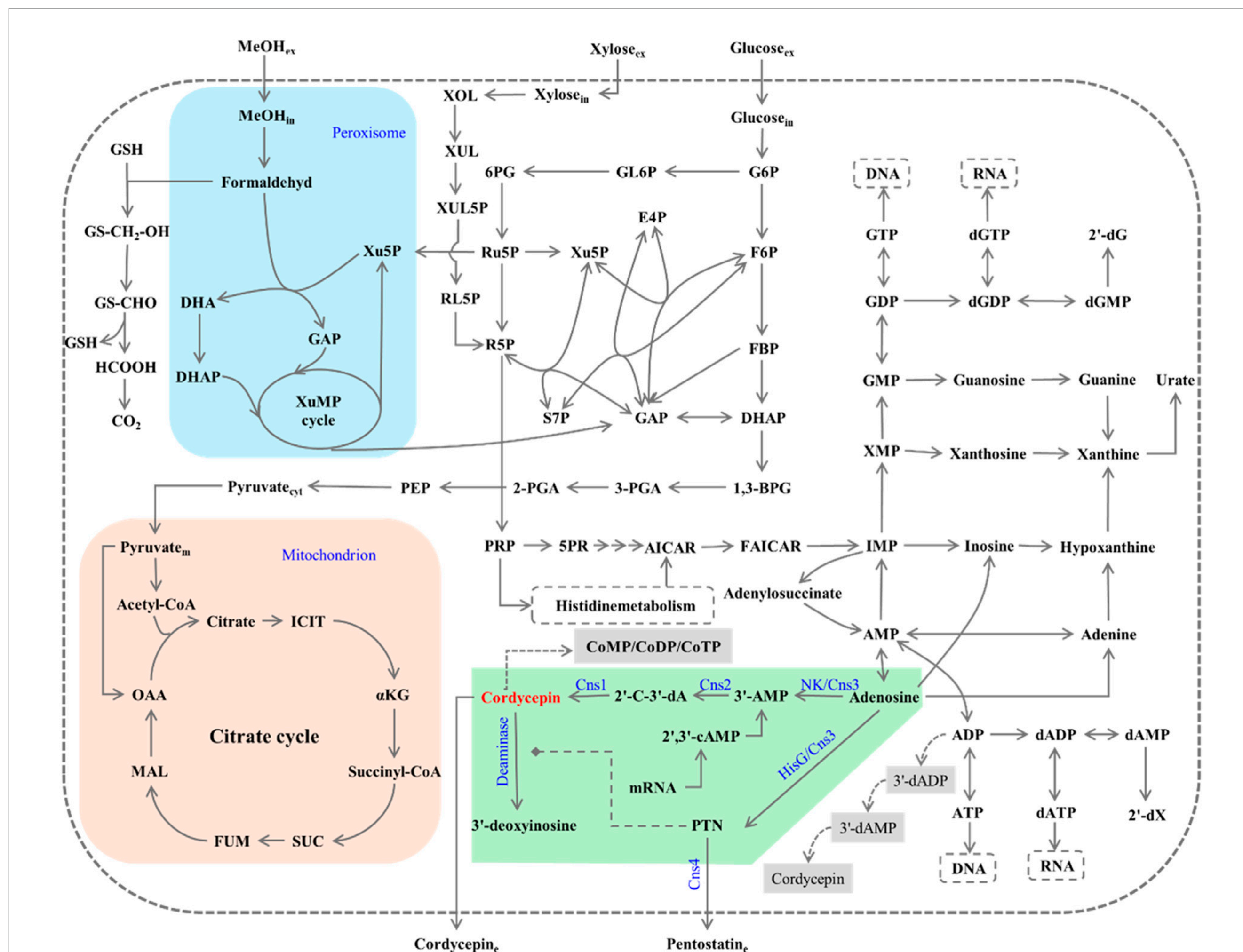

FIGURE 1
The metabolic pathways related to the biosynthesis of cordycepin in *Cordyceps militaris* and engineered microorganisms. GSH, glutathione; GS-CH_2-OH, S-(Hydroxymethyl)glutathione; GS-CHO, S-Formylglutathione; HCOOH, Formate; CO_2, Carbon dioxide; DHA, Dihydroxyacetone; DHAP, Dihydroxyacetone phosphate; GAP, Glyceraldehyde 3-phosphate; Xu5P, xylulose-5-phosphate; Ru5P, Ribulose 5-phosphate; R5P, Ribose 5-phosphate; XOL, Xylitol; XUL, D-Xylulose; XUL5P, D-Xylulose 5-phosphate; RL5P, D-Ribulose 5-phosphate; 6PG, 6-Phospho gluconate; GL6P, Glucono-1,5-lactone 6-phosphate; G6P, Glucose 6-phosphate; F6P, Fructose 6-phosphate; FBP, Fructose-1,6-bisphosphatase I; E4P, Erythrose 4-phosphate; S7P, Sedoheptulose 7-phosphate; 1,3-BPG, Glycerate 1,3-diphosphate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, Phosphoenolpyruvate; ICIT, Isocitrate; αKG, 2-Ketoglutaric acid; SUC, Succinate; FUM, Fumarate; MAL, Malate; OAA, Oxaloacetate; PRPP, 5-Phosphoribosyl diphosphate; 5PR, 5-Phosphoribosylamine; AICAR, 5′-Phospho-ribosyl-5-amino-4-imidazole carboxamide; FAICAR, 5′-Phosphoribosyl-5-formamido-4-imidazolecarboxamide; IMP, Inosine 5′-phosphate; XMP, Xanthosine 5′-phosphate; GMP, Guanosine 5′-phosphate; GDP, Guanosine 5′-diphosphate; GTP, Guanosine 5′-triphosphate; dGDP, 2′-Deoxyguanosine 5′-diphosphate; dGTP, 2′-Deoxyguanosine 5′-triphosphate; dGMP, 2′-Deoxyguanosine 5′-monophosphate; 2′-dG, 2′-Deoxyguanosine; AMP, Adenosine 5′-monophosphate; ADP, Adenosine 5′-diphosphate; ATP, Adenosine 5′-triphosphate; dADP, 2′-Deoxyadenosine 5′-diphosphate; dATP, 2′-Deoxyadenosine 5′-triphosphate; dAMP, 2′-Deoxyadenosine 5′-monophosphate; 2′-dX, 2′-Deoxyadenosine; 3′-dADP, 3′-Deoxyadenosine 5′-diphosphate; 3′-dAMP, 3′-Deoxyadenosine 5′-monophosphate; 3′-AMP, 3′-Adenylic acid; 2′-C-3′ -dA, 2′-carbonyl-3′ -deoxyadenosine; *CNS1*, Oxidordeuctase involved of cordycepin biosynthesis; *CNS2*, Metal dependent phosphohydrolase involved of cordycepin biosynthesis; 2′-3′-cAMP, 2′,3′-cyclic adenosine monophosphate; *NK/Cns3*, Cns3 contains an N-terminal nucleoside/nucleotide kinase; *HisG/Cns3*, Cns3 contains a C-terminal HisG family of ATP phosphoribosyltransferases; *Cns4*, Transporter proteins of Pentostatin; CoMP, Cordycepin monophosphate; CoDP, Cordycepin diphosphate; CoTP, Cordycepin triphosphate.

body cultivation and complex environmental responses during liquid fermentation. Factors like light, temperature, oxygen supply, redox state, and metal ions significantly affect yield, leading to substantial batch-to-batch variation (Zhao et al., 2020; Wang et al., 2022). Consequently, with the development of synthetic biology, alternative microbial cell factories such as *Saccharomyces cerevisiae* (Huo et al., 2021; Wang et al., 2024), unconventional yeasts *Komagataella phaffii* (Tan et al., 2023) and *Yarrowia lipolytica* (Duan et al., 2022; Song et al., 2023), and *Aspergillus oryzae* (Jeennor et al., 2023) have been developed in recent years for cordycepin production (Table 1). Some of these engineered strains surpass natural producers in terms of production intensity and give simpler downstream purification due to their defined culture media. Given the toxicity of cordycepin to host cells, improving cell tolerance to cordycepin stress and elucidating its underlying mechanisms are crucial for constructing efficient cell factories.

This review will compare and analyze the characteristics and advantages of different chassis cells cordycepin factories, discuss key targets affecting cordycepin yield based on elucidated biosynthetic

pathways, and propose feasible tools and strategies for simultaneously enhancing stress tolerance to cordycepin and the yield.

2 Biosynthesis pathways of cordycepin

The genome of *C. militaris* was sequenced in 2011 (Zheng et al., 2011). Subsequently, Xia et al. (2017) identified the key gene cluster responsible for cordycepin biosynthesis through comparative genomic analysis with *A. nidulans*. In their work, this gene cluster, designated as Cns1-4, proved pivotal in the biosynthetic pathway of cordycepin (Figure 1), whereas 3′-AMP serves as the substrate of dephosphorylation by Cns2, a metal-dependent phosphohydrolase, to form 2′-carbonyl-3′-deoxyadenosine (2′-C-3′-dA). This intermediate is then converted to cordycepin through a redox reaction catalyzed by Cns1, an oxidoreductase, and 3′-AMP can be produced from adenosine via the nucleotide kinase domain (NK) at the N-terminus of Cns3 or from 2′,3′-cAMP, a product of mRNA degradation that is ubiquitously present in various cells (Wongsa et al., 2020). Therefore, theoretically, the overexpression of Cns1 and Cns2 in a host cell can utilize endogenous 3′-AMP to achieve heterologous cordycepin production. The C-terminal HisG domain of Cns3 catalyzes the conversion of adenosine to pentostatin (PTN), an adenosine deaminase inhibitor that prevents the deamination and subsequent inactivation of cordycepin. Cns4, an ABC transporter, exports PTN extracellularly. This mechanism ensures that when intracellular concentrations of cordycepin become excessively high, cordycepin can be converted to the non-toxic 3′-deoxyinosine (3′-dI) form, thereby protecting the cells. This system is referred to as the Protector-Protégé feedback mechanism. Additionally, Xia et al. (2017) also found that Cns1 and Cns2 could form a functional protein complex localized to lipid droplets. Later, similar gene clusters with analogous functions to Cns1-4 were also identified in *C. kyushuensis* (designated as ck1-4) (Zhao et al., 2019) and *C. cicadae* (Liu et al., 2018).

In contrast, no similar gene clusters have been identified in *O. sinensis* (Xia et al., 2017). Transcriptomic analysis suggests that *O. sinensis* may rely on an alternative reductive metabolic pathway for cordycepin biosynthesis (Xiang et al., 2014) (lower right corner in Figure 1): AMP is converted to ADP by adenylate kinase (ADEK), then reduced to 3′-dADP by ribonucleotide reductase (NRDJ), followed by dephosphorylation to 3′-dAMP by ADEK, and finally converted to cordycepin through a dephosphorylation reaction catalyzed by 5′-nucleotidase (NT5E). However, conclusive experimental evidence is still lacking.

3 Natural cordycepin-producing fungus *Cordyceps militaris*

Despite the development of various microbial chassis cells as cordycepin-producing cell factories, some already achieving high production levels, *C. militaris* remains irreplaceable for cordycepin production for several reasons. Firstly, *C. militaris* demonstrates excellent biosafety, can be artificially cultivated (Kontogiannatos et al., 2021), and has a broad substrate spectrum, utilizing not only silkworm pupae, but also rice, barley, and xylose (Wongsa et al., 2020) as carbon sources. In countries like China, it has a long history of use as both food and medicine (Cui, 2015), leading to high social acceptance. Notably, as early as 2009 *C. militaris* was announced as a kind of safe food by Chinese government, providing regulatory support for the application and promotion of cordycepin products from *C. militaris*, a benefit that cordycepin products from yeast cell factories currently lack. Additionally, in 2016, the CRISPR-edited, browning-resistant *Agaricus bisporus* was approved by the US Department of Agriculture (USDA) (Waltz, 2016), suggesting that other mushrooms, including *C. militaris*, could potentially receive authorization for genetic editing in the future.

Secondly, *C. militaris* contains various bioactive molecules such as cordyceps polysaccharides, D-mannitol, N6-(2-hydroxyethyl)-adenosine (HEA), and carotenoids, etc. (Wu et al., 2021). Cordycepin, its signature active substance, can synergize with these compounds to target multiple sites, enhancing health and therapeutic effects. Cordycepin is also a key indicator for grading the quality of *C. militaris* fruiting bodies in China. Therefore, breeding *C. militaris* strains to increase cordycepin yield is of importance.

However, due to the lack of genetic editing tools, previous research mainly focused on optimizing fermentation strategies (solid, static, and submerged fermentation) for *C. militaris*, as reviewed previously by Wang et al. (2022). Recently, with the complete elucidation of the *C. militaris* genome and advances in molecular biology techniques, breeding methods for *C. militaris* have diversified. These methods now include the development of gene editing toolkit (Chen et al., 2018; Chen et al., 2022; Meng et al., 2022) and metabolic pathway optimization (Zhang et al., 2022), genome shuffling (Wang et al., 2017), and multi-omics approaches to identify key metabolic nodes.

Chen et al. (2022) successfully developed the CRISPR-Cas9-TRAMA system, achieving precise multiple gene editing and large DNA fragment deletions. Unlike exogenously synthesized gRNA, this system synthesizes gRNA endogenously using tRNA and *PtrpC* promoters. The polyethylene glycol-mediated transformation (PMT) was found superior to *Agrobacterium tumefaciens*-mediated transformation (ATMT) for genetic transformation. The plasmid includes the AMA1 sequence (Meng et al., 2022) from *A. nidulans* Glasgow strains, making it an episomal plasmid that can be eliminated by selective removal in the culture medium. The study also found that *C. militaris* can repair DNA through non-homologous end joining (NHEJ) or homology-directed repair (HDR). Future efforts to enhance genetic editing and metabolic modification in *C. militaris* should focus on elucidating key components influencing NHEJ and HDR, identifying neutral sites for gene (cluster) integration and expression, discovering efficient constitutive or inducible promoters (Lyu et al., 2022) (including AI-predicted promoters), and developing abundant selection markers (Lou et al., 2021) with more efficient transformation/screening methods.

Genome shuffling (Biot-Pelletier and Martin, 2014), combined with traditional physical (such as plasma, proton beam irradiation or UV) or chemical (such as nitrosoguanidine) mutagenesis (Masuda et al., 2011; Zhang et al., 2020), and adaptive laboratory evolution (ALE) (Mavrommati et al., 2022) remain effective breeding methods, though they often yield uncertain results and require substantial labor. Current methods for detecting cordycepin primarily rely on HPLC or thin-layer chromatography. There is a lack of colorimetric or fluorescent labeling methods for high-

throughput screening. Breakthroughs in this area could significantly enhance the efficiency of both rational and non-rational genetic engineering. One solution is to identify endogenous promoters able to linearly respond to cordycepin concentration as biosensors, converting different cordycepin yields into phenotypes such as growth rate variations which could easily be recognized. Besides, Omics research plays a crucial role in exploring cordycepin biosynthesis pathways, identifying key enzyme genes and metabolic nodes (Chen et al., 2020; Zhang et al., 2023; Chai et al., 2024).

4 Yeast cell factories for biosynthesis of cordycepin

4.1 *S. cerevisiae*, the first heterologous cordycepin expression platform

Cordycepin was first biosynthesized in the model organism *S. cerevisiae*, establishing the initial yeast cell factory for heterologous cordycepin expression (Xia et al., 2017; Huo et al., 2021). It was demonstrated that expressing only Cns1 and Cns2 is sufficient for cordycepin heterologous expression, while the yield was merely 137.27 mg/L even after fermentation optimization (Huo et al., 2021). By overexpressing the *cpdB* gene (3′-cyclic-nucleotide 2′-phosphodiesterase/3′-nucleotidase) from *Escherichia coli*, which converts 2′, 3′-cAMP to 3′-AMP, cordycepin concentration was significantly increased (Wang et al., 2024). This indicates that 3′-AMP in *S. cerevisiae* is similarly to that in Cordyceps fungi, derived via RNA degradation pathways, and that Cns3 is not necessary for heterologous cordycepin production. This finding has also been validated in other yeast cell factories.

Subsequently, Wang et al. (2024) improved cordycepin yield to 725.16 mg/L through rational metabolic engineering. Their study specifically compared the catalytic efficiency of Cns1/2 homologs from the marine fugus *Emericellopsis atlantica* and found that codon-optimized EA1/2 from the marine fungus increased cordycepin titer by 18.6% compared to Cns1/2, which underscores the importance of enzyme activity in the yeast host for cordycepin yield and the potential for mining potential sources of cordycepin synthesis genes from microorganisms in unique environments or through enzyme evolution and screening.

In metabolic engineering, enhancing the pentose phosphate pathway and purine synthesis pathway genes, including *ZWF1* (glucose-6-phosphate dehydrogenase), *PRS4* (ribose phosphate diphosphokinase), and *ADE4* (amidophosphoribosyl transferase), significantly increased cordycepin yield (Wang et al., 2024). Overexpressing Cns1-2 in *S. cerevisiae* also upregulated these genes at the transcriptional level (Huo et al., 2021), indicating that synthesis of PRPP (5′-phosphoribosyl diphosphate) and the diversion of carbon flux into purine metabolism are critical nodes for improving cordycepin synthesis, partially consistent with previous findings in *C. militaris* (Chen et al., 2020).

Notably, deleting the *ADO1* gene significantly increased cordycepin yield (Wang et al., 2024). One possible explanation is that this deletion enhances host tolerance to cordycepin stress, providing a novel approach to increasing cell factory yields, suggesting the tolerance of the chassis cells to toxic products may determine yield limits. Cordycepin can be phosphorylated to cordycepin triphosphate (Hawley et al., 2020), which strongly inhibits RNA synthesis and causes cellular toxicity (Holbein et al., 2009). Deleting *ADO1* may disrupt adenosine phosphorylation to AMP, potentially affecting phosphorylation levels and ratios of cordycepin (Wang et al., 2024). Further evidence, such as measuring cordycepin phosphorylation levels in yeast or identifying endogenous enzymes involved in cordycepin phosphorylation, could validate this hypothesis.

4.2 Non-conventional yeasts: *K. phaffii* and *Y. lipolytica*

Non-conventional yeasts have recently emerged as effective cell factories for the production of various natural products (Rebello et al., 2018). Cordycepin-producing cell factories have been successfully developed in the non-conventional yeasts *K. phaffii* (Tan et al., 2023) and *Y. lipolytica* (Duan et al., 2022; Song et al., 2023). These systems utilize codon-optimized Cns1 and Cns2 genes derived from *C. militaris*, achieving yields of 2.68 g/L in shake flasks and 4,362.54 mg/L in fermenters, respectively.

In the construction and optimization of the *Y. lipolytica* cordycepin cell factory, various molecular biology strategies were employed (Duan et al., 2022; Duan X. Y. et al., 2023; Song et al., 2023), including multi-copy integration of the key synthesis enzymes Cns1-2 at the rDNA locus, promoter optimization, and different expression methods for key enzymes (linker/2A peptide/RIAD-RIDD protein scaffold). Typically, exogenous gene expression in engineered strains using free plasmids results in unstable yields due to uneven plasmid distribution during cell division, not mentioning the need for antibiotics to maintain plasmid presence (Friehs, 2004). In contrast, integration at loci such as rDNA (Le Dall et al., 1994), delta sequences (Sakai et al., 1990), or IS elements (Lee et al., 2016) could ensure both the stability and multi-copy integration.

To optimize metabolic pathways, several genes in *Y. lipolytica* were overexpressed including *GLK1* (Glucokinase) and phosphoglucomutases (*PGM1*) to enhance the pentose phosphate pathway (PPP) and PRPP production (Duan X. Y. et al., 2023). Overexpression of adenylate succinate synthase (*ADE12*) and adenylosuccinate lyase (*ADE13*) upregulated the purine synthesis pathway. Furthermore, the overexpression of 3′-phosphoglycerate kinase (*PGK*) and pyruvate kinase (*PK*) effectively increased intracellular ATP levels, significantly boosting cordycepin production (Song et al., 2023). This indicates that PPP, purine synthesis pathways (affecting precursor supply for cordycepin synthesis), and ATP supply and consumption are crucial metabolic targets for cordycepin biosynthesis.

In *K. phaffii*, a two-stage fermentation process using glycerol/methanol was employed, with the inducible promoters *AOX1p* and *FLD1p* expressing Cns1 and Cns2 from *C. militaris*, respectively (Tan et al., 2023). This approach, achieving inducible expression of cordycepin in a cell factory for the first time, spatially separated the cell growth phase (glycerol) from the cordycepin production phase (methanol), avoiding too early accumulation of cordycepin that might hinder cell growth. Transcriptome analysis revealed downregulation of genes related to methanol assimilation and

dissimilation, peroxisome biogenesis, and the pentose phosphate pathway, providing targets for further strain improvement. The broad substrate utilization spectrum of *K. phaffii*, especially its ability to utilize methanol as a sole carbon and energy source (Ata et al., 2021; Zha et al., 2023), positions it competitively in controlling cordycepin fermentation costs and downstream product purification.

As in *Cordyceps militaris*, the yield of cordycepin in yeast cell factories can also be increased through process engineering optimization. For example, the addition of adenine has been shown to significantly enhance cordycepin production in all reported yeast cell factories, whereas adding adenosine can only partially increase the yield in the engineered *Y. lipolytica* (Song et al., 2023). Furthermore, optimizing the type and concentration of carbon and nitrogen sources, as well as the C/N ratio, has been crucial for the engineered *Y. lipolytica* (Duan X. Y. et al., 2023). In the case of engineered *K. phaffii*, the initial pH and methanol concentration in flask fermentation have significant effects on the final productivity (Tan et al., 2023).

4.3 Optimization strategies for yeast cell factories

Based on a comparative analysis of three yeast cell factories, the following optimization strategies are proposed.

4.3.1 Enhancing host stress tolerance and establishing high-throughput screening platforms

The superior cordycepin synthesis capabilities in *Y. lipolytica* and *K. phaffii* compared to *S. cerevisiae* are largely attributed to their greater tolerance to cordycepin stress. In *Y. lipolytica*, cordycepin concentrations up to 3.0 g/L (Duan et al., 2022) did not affect final biomass, revealing the importance of considering host stress tolerance when generating toxic products. Techniques of genome evolution (Huang C. et al., 2022) like Synthetic Chromosome Rearrangement and Modification by *LoxP*-mediated Evolution (SCRaMbLE) (Cheng et al., 2024) can potentially enhance both cordycepin stress tolerance and production yield. NHEJ-mediated genomic library construction in *Y. lipolytica* can also introduce numerous *LoxP* sites (Bai et al., 2021). Additionally, adaptive laboratory evolution (Fernandes et al., 2023) remains effective; however, mutants with improved cordycepin production ability lack visible phenotypes or growth-coupled traits, making them difficult to screen. Therefore, efficient screening methods based on high-throughput platforms are critical. Traditional methods relying on HPLC are too laborious and costly to efficiently identify targets from thousands of (or much more) mutants.

One widely implemented strategy in cell factories is the use of transcription factor (TF)-based biosensors. For instance, the fatty acid and phospholipid regulator FapR and its operator *fapO* from *Bacillus subtilis* have been extensively studied and applied in both prokaryotic and eukaryotic systems as a malonyl-CoA sensor (Zhang and Shi, 2021). The allosteric conformational changes in transcription factors (TFs) when binding a specific metabolite leads to their binding to or dissociation from the operator region, thus regulating transcription. Endogenous transcription factors that respond to cordycepin concentration can be identified through transcriptome data mining of the host cell. Additionally, artificial transcription factors can be developed using reported cordycepin-binding receptors (Nakamura et al., 2015). These artificial TFs can be fused with reporter gene modules (such as fluorescent proteins or antibiotic resistance genes) to establish intracellular cordycepin biosensors.

4.3.2 Cordycepin transport and deamination

Adenosine cannot freely permeate biological membranes and its transport relies on selected protein carriers (Pastor-Anglada and Perez-Torras, 2018), whereas its structural analogue cordycepin has been reported to be abundantly present extracellularly in various existing cell factories, indicating the presence of endogenous cordycepin transport proteins. Future work should assess intracellular versus extracellular cordycepin distribution to identify and enhance expression of potential cordycepin export proteins.

Deamination is a detoxification mechanism used by cells to neutralize cordycepin (Chen et al., 2023), but in cell factory fermentation for cordycepin production, deamination should be avoided. The deamination issue observed in *K. phaffii* (Tan et al., 2023) suggests the presence of endogenous deaminases that recognize cordycepin, necessitating study on the mechanism and stringent fermentation control.

4.3.3 Flocculation and fermentation-separation coupled systems

Given cordycepin's toxicity, timely removal from the fermentation broth can alleviate product feedback inhibition and maintain cell activity. Coupled fermentation-separation systems, established in *C. militaris* (Guan et al., 2019), could be adapted for yeast, especially using self-flocculating yeasts to enhance efficiency. *S. cerevisiae* can achieve flocculation through the function of *FLO* genes (Verstrepen et al., 2003), and expressing the *FLO1* gene from *S. cerevisiae* in *K. phaffii* has shown some degree of flocculation (Sae-Tang et al., 2023). Flocculation can also increase cell density, reinforcing high-density fermentation for yield improvement.

4.3.4 Compartmentalized expression strategies and host-specific metabolic engineering

In *C. militaris*, Cns1/2 form complexes localized on lipid droplets (Xia et al., 2017), a phenomenon also observed in heterologous expression in *S. cerevisiae* (Wang et al., 2024). Although no such information was available for non-conventional yeasts, compartmentalization can mitigate toxicity of the product. *Y. lipolytica*'s natural advantage in lipid production (Lazar et al., 2018) might partially explain its high yield if Cns1-2 localizes on lipid droplets. *K. phaffii*, under methanol induction, efficiently expands peroxisomes, suggesting a potential for organelle-targeted enzyme localization via signal peptide fusion (Ye et al., 2024).

For substrate conversion, similar metabolic modules and key pathways are followed across host cells, necessitating PPP and purine synthesis pathway enhancement to improve 3′-AMP supply. Complex feedback inhibition of enzymes like PRPP synthetase could be addressed by enzyme mutation or introducing high-activity enzymes from other species (Huang Z.

et al., 2022). ATP supply and consumption also require consideration. Inducible promoters can temporally separate growth and production phases. Tailored metabolic pathway optimization strategies should be designed for different substrates, like enhancing methanol metabolism in *K. phaffii* or incorporating synthetic, more efficient methanol utilization pathways to supplement with the current Xu-5-P cycle (Antoniewicz, 2019).

5 Other cell factories

Synthetic pathways for cordycepin production have been successfully engineered in *A. oryzae* (Jeennor et al., 2023), where the integration of Cns1-2 from *C. militaris* under constitutive promoters resulted in a productivity of 564.64 ± 9.59 mg/L/day and 98% cordycepin secretion. Similar to *C. militaris*, supplementation with adenine, adenosine, or glycine could enhance production, with adenine being the most effective precursor. This research provides a food-grade platform for heterologous expression of cordycepin and accommodates a broader range of carbon sources.

It is noteworthy that the aforementioned hosts predominantly consist of eukaryotic cells, with limited progress observed in prokaryotic platforms. Despite advantages in growth rates and heterologous protein expression at a high titer, the complex structure of Cns1 hampers its soluble expression in prokaryotic microbes like *E. coli* (Xia et al., 2017) and *B. subtilis* (Duan et al., 2022).

6 Summary

In conclusion, the advancement of synthetic biology promises the development of more diverse chassis cells for cordycepin production, expanding its application scenarios. Non-conventional yeast cell factories hold great potential for achieving high yields and offer advantages in separation and purification processes following fermentation. Investigating the stress mechanisms of cordycepin in host cells and enhancing stress tolerance through genetic and process engineering is crucial for yield improvement. The task of establishing high-throughput screening platforms using biosensing systems for cordycepin production is urgent. Further research is needed to explore genetic elements responsible for cordycepin synthesis from broader sources, with mutation and screening facilitating compatibility with the chosen chassis cell.

Author contributions

XL: Writing–original draft, Visualization. RJ: Writing–original draft. SW: Conceptualization, Writing–original draft. CL: Writing–original draft. YX: Conceptualization, Funding acquisition, Writing–review and editing. SL: Conceptualization, Funding acquisition, Writing–review and editing. QL: Writing–original draft, Writing–review and editing, Conceptualization, Funding acquisition. LW: Conceptualization, Funding acquisition, Project administration, Writing–review and editing.

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Conflict of interest

Authors YX and SL were employed by Dalian SEM Bio-Engineering Technology Co., Ltd., while QL and LW are conducting postdoctoral research at the postdoctoral workstation of Dalian SEM Bio-Engineering Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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