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A new *Clonostachys* Sp. ZBS49 filamentous fungus with high production of betulinic acid and its inhibitory effect on liver cancer cells

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Abstract

Background Triterpenoid compounds such as betulinic acid (BA) and oleanolic acid (OA) exhibit considerable pharmacological activities. However, their current production primarily relies on plant extraction and chemical synthesis, methods that are often plagued by low efficiency, complex extraction processes, and environmental concerns. Microbial-based synthesis has emerged as one of the most effective approaches for producing BA and OA.

Results This study presented the first identification of filamentous fungal strains efficiently synthesizing both BA and OA. The strain ZBS49 is a newly identified species of *Clonostachys* isolated from *Gleditsia japonica* Miq. (showing 99.82% sequence identity), produced 47.7 mg/L of BA. The strain XJ1-1, characterized as *Colletotrichum gloeosporioides* and isolated from *Cannabis sativa* L., yielded 65.76 mg/L of OA. After optimizing the culture medium and cultivation conditions, the yields of ZBS49 and XJ1-1 increased to 288.97 and 86.14 mg/L, representing improvements of 506% and 31%, respectively. Furthermore, we discovered that the BA extract of the ZBS49 strain significantly inhibited hepatocellular cancer cells (SMMC-7721 and HepG2) in a dose-dependent manner, with a minimum inhibitory concentration of 70 μM. Genomic analysis of *Clonostachys* sp. ZBS49 elucidated that the presence of 16 putative genes was related to triterpenoid biosynthesis and 6 distinct terpene biosynthetic gene clusters. Among the 145 CYP450, 5 genes involved in C-28 oxidation were predicted.

Conclusions This research underscores the effectiveness of filamentous fungi as a biotechnological platform for the efficient production of BA and its derivatives, highlighting their potential applications in cancer therapy. Furthermore, these findings provide valuable genetic resources and establish a robust technical and theoretical framework for utilizing ZBS49 as a microbial platform for the biosynthesis of triterpenoids.

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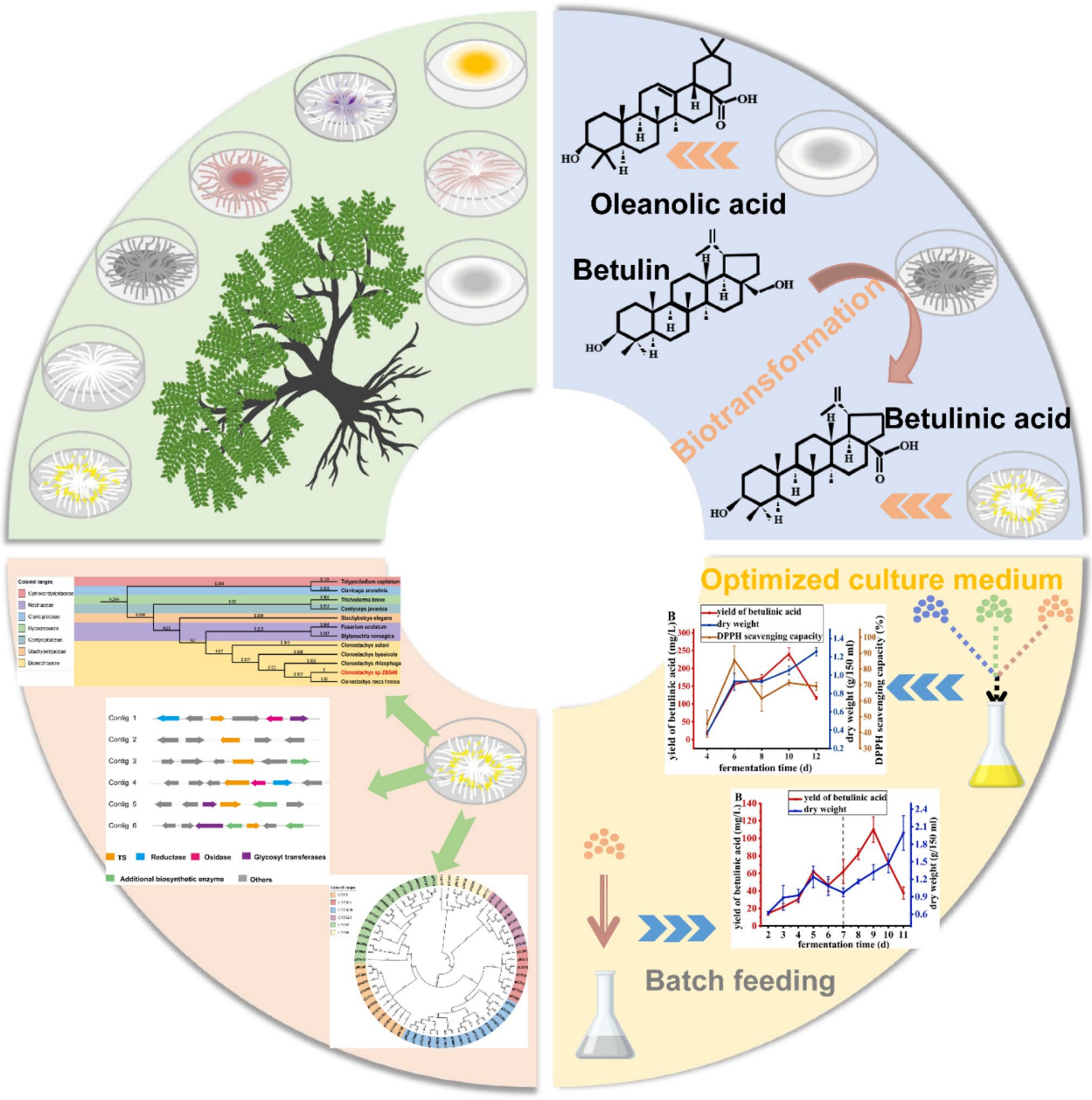
Graphical abstract



Keywords Filamentous fungi, Betulinic acid, Fermentation system, *Clonostachys* Sp., Genome, Tumor suppression

Background

Triterpenoid compounds, such as betulinic acid (BA) and oleanolic acid (OA), are characterized by significant structural diversity and broad biological activities, including anticancer, antioxidant, and antiviral properties [1–6]. BA and OA have attracted considerable commercial and academic research attention due to their low cytotoxicity toward normal cells and their hepatoprotective effects [7, 8]. The extraction BA from *Zizyphus Juazeiro* barks using a focused microwave was investigated, with a maximum yield of 3.33 mg/g achieved [9]. Similarly, Medina et al. showed OA achieving a 13 mg/g yield from Fructus *Ligustri Lucidi* [10]. Despite various optimization efforts, plant extraction faces challenges

such as low natural abundance, resource depletion, and lengthy processing times. Nonetheless, employing microorganisms as platforms for the biosynthesis of terpenoids via synthetic biology and metabolic engineering offers a more sustainable production strategy with promising applications [11, 12].

Endophytic fungi inhabit plant tissues, significantly promote plant growth, and enhance stress tolerance through their interactions with host plants [13, 14]. Due to their long-term co-evolution with host plants, these fungi are capable of synthesizing metabolites that closely resemble or are identical to those of their plant hosts, many of which possess valuable medicinal properties [15, 16]. Consequently, endophytic fungi have been extensively utilized to produce various natural products, including paclitaxel, podophyllotoxin, and huperzine A [17–19]. Furthermore, these fungi have emerged as promising sources of secondary metabolites featuring novel structures and significant biological activities, such as antimicrobial, insecticidal, anticancer, plant hormone, and antioxidant effects [20–23]. Endophytic fungi also exhibit unique biotransformation capabilities, which enable the production of complex natural product scaffolds. This characteristic has proven valuable in discovering novel chemical structures, drug synthesis, and the degradation of environmental pollutants [24–26]. Therefore, utilizing endophytic fungi to synthesize BA or OA represents a promising green economic strategy. Notably, *Phomopsis* sp. is the first endophytic fungus isolated from *Diospyros carbonaria* Benoist capable of producing BA; however, the yield produced by this fungus is considerably low, failing to meet the requirements for industrial production [27]. Similarly, Feng et al. demonstrated that *Cunninghamella blakesleeana* can transform betulin (BT) into at least five products, with BA being the predominant compound [28]. Additionally, *Inonotus obliquus* has been shown to produce BA [29], with BA yield increasing by 200% and 429.5% upon adding 1.0 g/L OA and 45 mg/L fungal inducer, respectively [30].

The triterpene biosynthesis pathway comprises the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways. Starting with 2,3-oxidosqualene as a precursor, β-amyrin synthase and lupeol synthase catalyze the formation of β-amyrin and lupeol, respectively. These intermediates are then converted into OA and BA by combining cytochrome P450 (CYP450) oxidases and reductases [31, 32]. Tang et al. constructed a biosynthetic pathway for BA in *Saccharomyces cerevisiae* by integrating multiple strategies, including enhancing the BA synthesis pathway, dynamically inhibiting competing pathways, boosting the supply of acetyl-CoA and NADPH, reconstructing the BA synthesis pathway in peroxisomes, and optimizing the fed-batch fermentation process, the final BA titer reached 682.29 ± 8.16 mg/L [32]. To construct an efficient OA-producing strain, Cheng et al. also employed multiple strategies, including regulating rate-limiting steps using inducible promoters, amplifying precursor pools, pathway reconstruction, and constructing diploid strains. As a result, the OA production reached 4.07 g/L in a 100-liter bioreactor [33].

In this study, endophytic fungi *Clonostachys* sp. ZBS49 and *Colletotrichum gloeosporioides* XJ1-1 were isolated from 171 endophytic fungal strains. By optimizing culture conditions, efficient production of the triterpenoid compounds BA and OA was successfully achieved, representing the highest BA and OA yield documented among filamentous fungi. Moreover, in vitro assays demonstrated that BA extracted from ZBS49 exhibited a significant dose-dependent inhibitory effect on liver cancer cells. Genomic analysis further identified genes and gene clusters associated with triterpenoid biosynthesis in ZBS49. This study provides a robust microbial biosynthesis platform, offering genetic resources and technical insights for the bioproduction of high-value triterpenoids. It also lays a solid theoretical and practical foundation for using BA in cancer cell therapy.

Methods

Cultivation of strains capable of synthesizing and transforming triterpenes

Previous studies isolated 171 endophytic fungal strains with secondary metabolite biosynthetic capabilities, including 20 strains from wild soybean, 50 from *Gleditsia japonica*, and 101 from *Cannabis sativa*. L. These endophytic fungi were inoculated onto solid media and cultured at 28 °C in a constant temperature incubator for 7–10 days. Subsequently, the fungal cultures were transferred into 250 mL Erlenmeyer flasks containing 150 mL of liquid medium. In screening strains capable of converting BT to BA, 2 mL aliquots of BT substrate stock solution were added to the liquid culture, yielding a final concentration of 42.67 mg/L. Fermentation without the addition of BT served as the blank control. All cultures were incubated in the dark at 28 °C with shaking at 150 rpm for 10 days.

Sample preparation and HPLC detection of triterpenoid content

The fungal mycelium was isolated using a centrifuge at 4000 rpm for 5 min, then dried at 60 °C and concentrated with a rotary evaporator. The concentrated mycelium was freeze-dried, ground into a powder, and immersed in anhydrous ethanol at a 1:20 ratio overnight at room temperature. Ultrasonic extraction was conducted for 2 h at 25 °C. The supernatant was collected, dried, and reconstituted with methanol for HPLC analysis. The BA and OA content were calculated as follows: $Y = 3*10^6$-2349.6, $R2 = 0.9994$; $Y = Y = 1*10^7$+33,446, $R2 = 0.9993$.

The HPLC system utilized was a Waters 600-717-2478 chromatographic system equipped with the HiQ sil C18 5 μm column (4.6 × 250 mm), employing a mobile phase of acetonitrile and water in a 9:1 ratio, a flow rate of 1.0 mL/min, the injection volume was 20 μL, and a detection wavelength set at 210 nm [34, 35].

LC-M analysis of ZBS49 mycelial extracts

LC-MS analysis was conducted using a HPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. Chromatographic separation was achieved on a Phenomenex Kinetex C18 column (2.1 × 50 mm, 2.6 μm) maintained at 25 ℃. The mobile phases consisted of (A) 0.01% acetic acid in water and (B) isopropanol: acetonitrile (1:1, v/v). The injection volume was 2 μL, and the autosampler temperature was set at 4 ℃.

Mass spectrometry was performed in information-dependent acquisition (IDA) mode, with full-scan MS (resolution: 60,000) and MS/MS (resolution: 15,000) scans. Electrospray ionization (ESI) parameters were optimized as follows: sheath gas, 50 arb; auxiliary gas, 15 arb; capillary temperature, 320 ℃; vaporizer temperature, 350 ℃; sweep gas, 1 arb. The spray voltage was set at − 3.4 kV, and stepped normalized collision energy (SNCE) was applied at 20, 30, and 40 eV.

Optimization of fermentation conditions

The endophytic fungal strains ZBS49 (producing BA) and XJ1-1 (producing OA) were inoculated into 6 different liquid media: Potato Flour Immersion Medium, Soybean Powder Culture Medium, Improved Martin Medium, Potato Culture Medium, Medicinal Fungal Culture Medium, and High-Salt Chahler Medium (Supplementary Table 2). After incubating in the dark for 10 days, samples were then taken to measure biomass and triterpene yields to identify the optimal culture medium. Subsequently, based on comparing BA or OA production and biomass accumulation under different culture conditions, medium components were optimized, including the optimal carbon source, nitrogen source, and pH. After identifying the optimal medium and culture conditions, fermentation time was further optimized by sampling at days 4, 6, 8, 10, and 12 of fermentation. Focused analysis was performed on the yield of BA and OA, along with changes in biomass, pH, residual sugar content, and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the fungal strains ZBS49 and XJ1-1.

Determination of DPPH free radical scavenging activity in fermentation broths of BA- and OA-producing strains

The DPPH radical scavenging assay was performed as follows: 400 μL of fungal mycelial extracts (at varying concentrations) were mixed with 400 μL of 1.5 mmol/L DPPH solution. The mixture was vortexed thoroughly and incubated in darkness at room temperature for 30 min. The absorbance at 517 nm was measured and recorded as A_{sample}.

For control measurements, 400 μL of 95% ethanol was substituted for the DPPH solution and mixed with 400 μL of methanol, with the absorbance recorded as $A_{control}$. The blank measurement consisted of 400 μL DPPH solution reacted with 400 μL methanol, with absorbance recorded as A_{blank}.

Inhibition of hepatocellular carcinoma cells by ethanol extract of BA synthetic strain *Clonostachys* sp. ZBS49

SMMC-7721 and HepG2 cells were cultured in 96-well plates and incubated for 24 h at 37 °C. Various concentrations of BA were then introduced, and cell viability was evaluated using the Calcein AM/PI assay. Cells were treated with BA for wound healing assessments and observed under a microscope. Apoptosis was evaluated after 24 h of BA treatment using Annexin V-FITC/PI staining followed by flow cytometry.

Genome analysis of *Clonostachys* sp. ZBS49

The genome of *Clonostachys* sp. ZBS49 was sequenced using short-read Illumina and long-read PacBio platforms by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). An Illumina HiSeq platform constructed the genomic library, and assembly was completed with SOAPdenovo2 to create optimal contigs, which were scaffolded based on paired-end and overlap relationships. Gene prediction was conducted using Maker2, SNAP, GeneMark-ES, and Augustus; annotations were performed via BLAST against various databases, including COG, GO, KEGG, NR, and SwissProt. Carbohydrate-active enzymes (CAZymes) were identified using the CAZy database, and cytochrome genes were annotated with the fungal CYP450 enzyme database. Secondary metabolite biosynthetic gene clusters were predicted using AntiSMASH 7.0.0 and manually verified. Phylogenetic analysis categorized ZBS49 within the genus *Clonostachys*, revealing its evolutionary relationships with related Hypocreales species. Orthologous gene clustering was performed using Ortho Finder and MCL clustering, and phylogenetic trees were constructed for each orthogroup, resulting in a rooted species tree inferred through the STRIDE algorithm.

Results

Screening of strains with the function of transforming BA, synthesizing BA or OA

To obtain an endophytic fungal chassis for the efficient synthesis of BA and OA, we screened 171 endophytic fungal strains derived from triterpene-producing plants in this study. Among these, 43 strains were identified as capable of converting BT to BA, 43 strains were capable

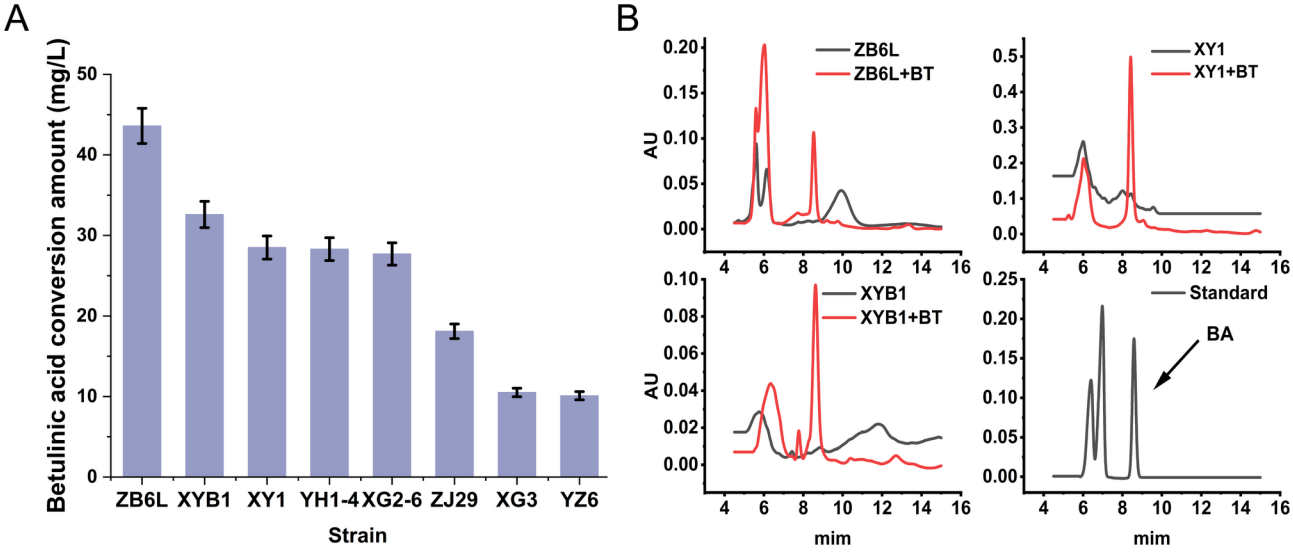


Fig. 1 Comparison of the amount of BA conversion of endophytic fungi with liquid chromatograms before and after biotransformation. Note: **A**: Amount of BA conversion of 8 endophytic fungi, **B**: Liquid chromatograms of ZB6L, XYB1, XY1 fermented alone versus after addition of BT (ZB6L + BT, XYB1 + BT, XY1 + BT)

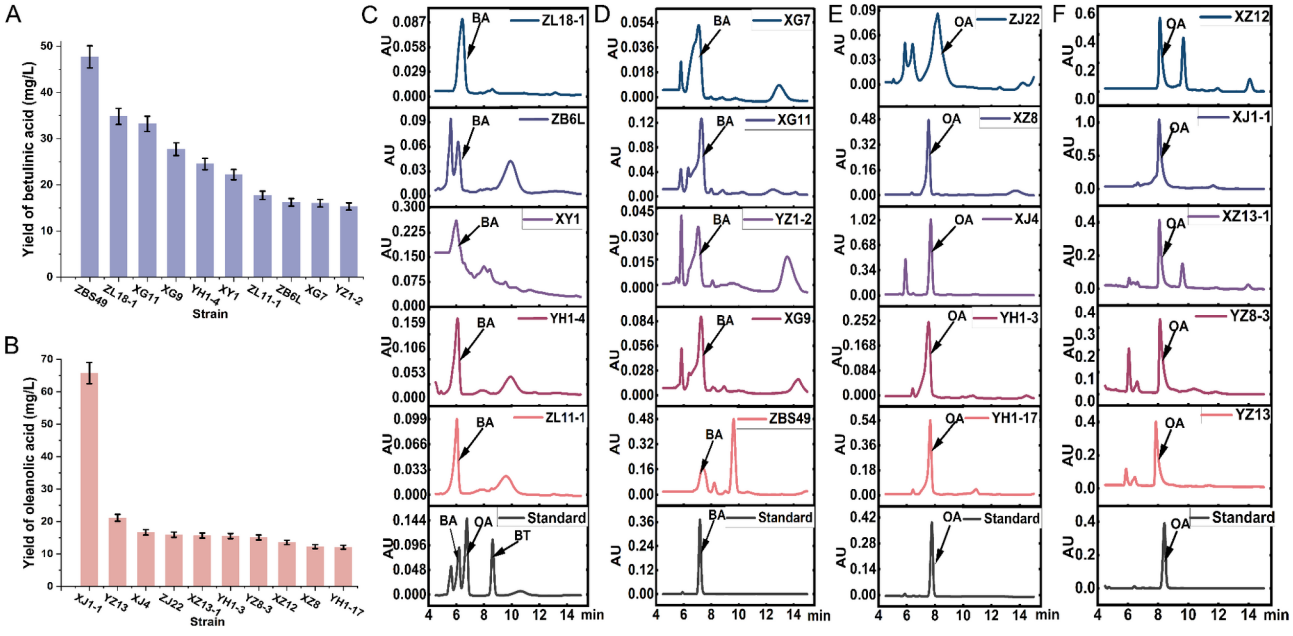


Fig. 2 Distribution of BA and OA in endophytic fungi and their analysis by HPLC. Note: **A**: BA yield in endophytic fungi, **B**: OA yield in endophytic fungi, **C**: strains ZL18-1, ZB6L, XY1, YH1-4, ZL11-1, **D**: strains XG7, XG11, YZ1-2, XG9, ZBS49, E: strains ZJ22, ZX8, XJ4, YH1-3, YH1-17, F: strains XZ12, XJ1-1, XZ13-1, YZ8-3, YZ13

of synthesizing BA, and 41 strains were capable of synthesizing OA. Among the strains tested, eight were able to convert BT to BA at levels exceeding 20 mg/L, with strain ZB6L exhibiting the highest conversion rate of 43.6 mg/L (Fig. 1). Additionally, ten strains produced more than 15.30 mg/L of BA, with ZBS49 generating the highest amount at 47.7 mg/L (Fig. 2A, C). Moreover, ten strains produced over 12.06 mg/L of OA, with strain XJ1-1 demonstrating a remarkable yield of 65.76 mg/L (Fig. 2B, C). The internal transcribed spacer (ITS) analysis revealed that ZBS49 exhibits 99.82% sequence identity with *Clonostachys* sp., highlighting its potential as a novel strain for synthesizing BA and its derivatives. Furthermore, LC-MS analysis of the mycelial extract from ZBS49 confirmed the significant presence of BA and various metabolites with potential medicinal and agricultural applications, including methyldazone, phenothiazine, and diltiazem. These findings highlight the promising

value of ZBS49 for diverse applications across multiple fields (Supplementary Table 3).

Optimization of fermentation conditions of strains ZBS49 and XJ1-1

The composition and conditions of culture media play a critical role in influencing the growth dynamics of fungal mycelium and the yield of metabolic products. To enhance the production of BA and OA, we first investigated the impact of 6 different culture media on the yields of strains ZBS49 and XJ1-1 under parallel conditions. (Fig. 3A, B). The results indicate that the mycelia of both fungi are capable of growing on all media except Czapek's high-salt medium. The production of BA by ZBS49 in soybean meal medium achieved its highest level at 139.13 mg/L, which is 2.92 times greater than that obtained in the PDB medium. Meanwhile, the yield of BA by XJ1-1 in the soybean meal medium was recorded at 30.67 mg/L, slightly lower than the yield in the PDB medium. Although the OA yield of strain XJ1-1 is higher when using the PDB medium, the complex preparation process of the PDB medium does not meet the requirements for industrial production. In contrast, soybean meal medium simplifies the process and is more conducive to large-scale fermentation applications. Therefore, soybean meal medium was selected for further research.

In the present study, we optimized fermentation conditions for strains ZBS49 and XJ1-1 via single-factor analysis. For strain ZBS49, the optimal culture parameters were determined to be 10 g/L sucrose, 10 g/L soybean meal extract, and an initial pH of 6 (Fig. 3C, E, G). For strain XJ1-1, the optimal conditions comprised 20 g/L

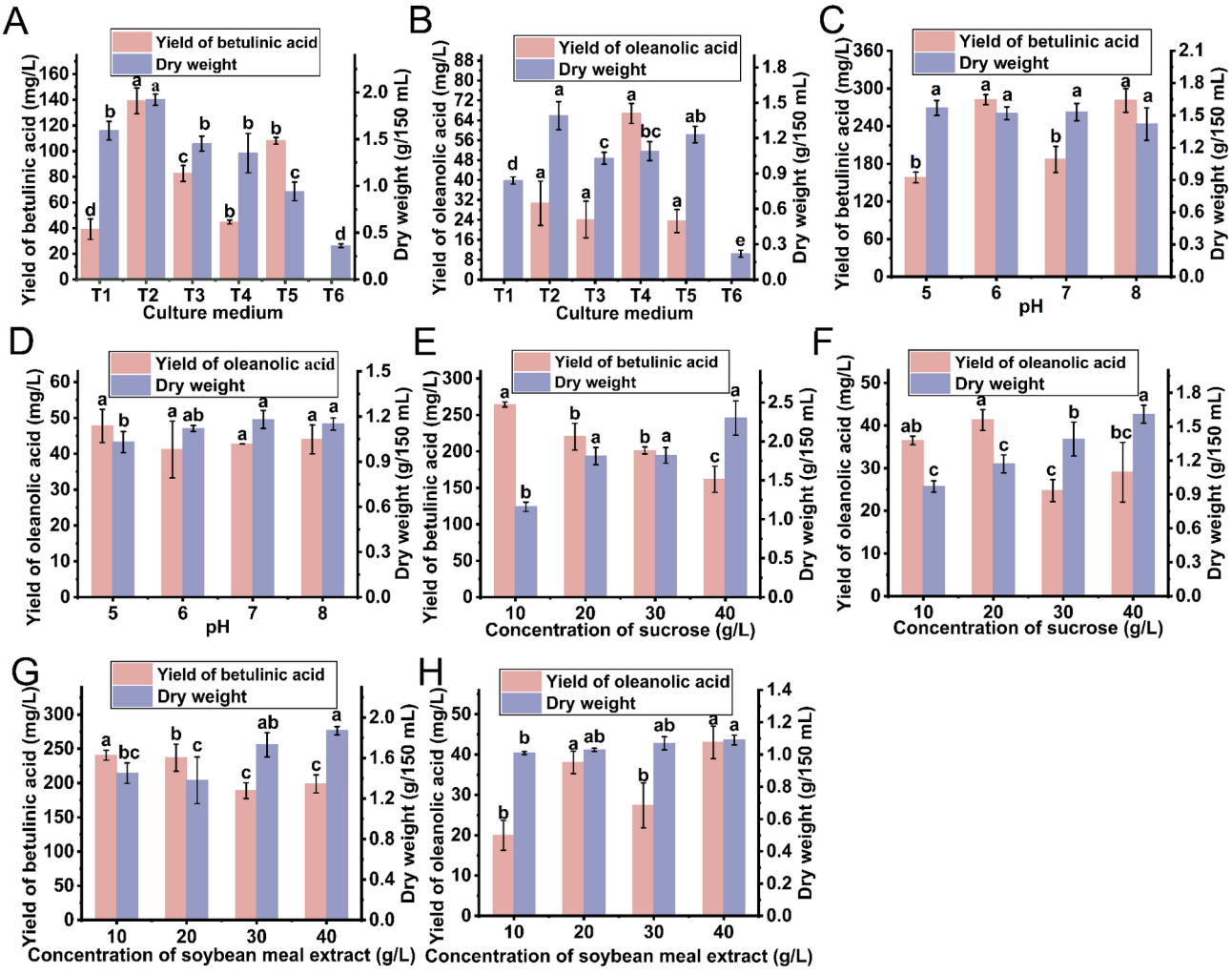


Fig. 3 Effects of fermentation conditions on growth and triterpene yield of strains. Note: **A-B**: Impact of media on growth and triterpenoid yield, T1: Potato powder culture medium, T2: soybean meal medium, T3: Modified Martin's medium, T4: PDB medium, T5: Medicinal fungi medium, T6: Czapek's high-salt medium; **C-D**: Effect of pH on growth and triterpenoid production in strains ZBS49 and XJ1-1; **E-F**: Influence of sucrose concentration on growth and triterpenoid production in strains ZBS49 and XJ1-1; **G-H**: Effects of soybean meal extract concentration on growth and triterpenoid production in strains ZBS49 and XJ1-1

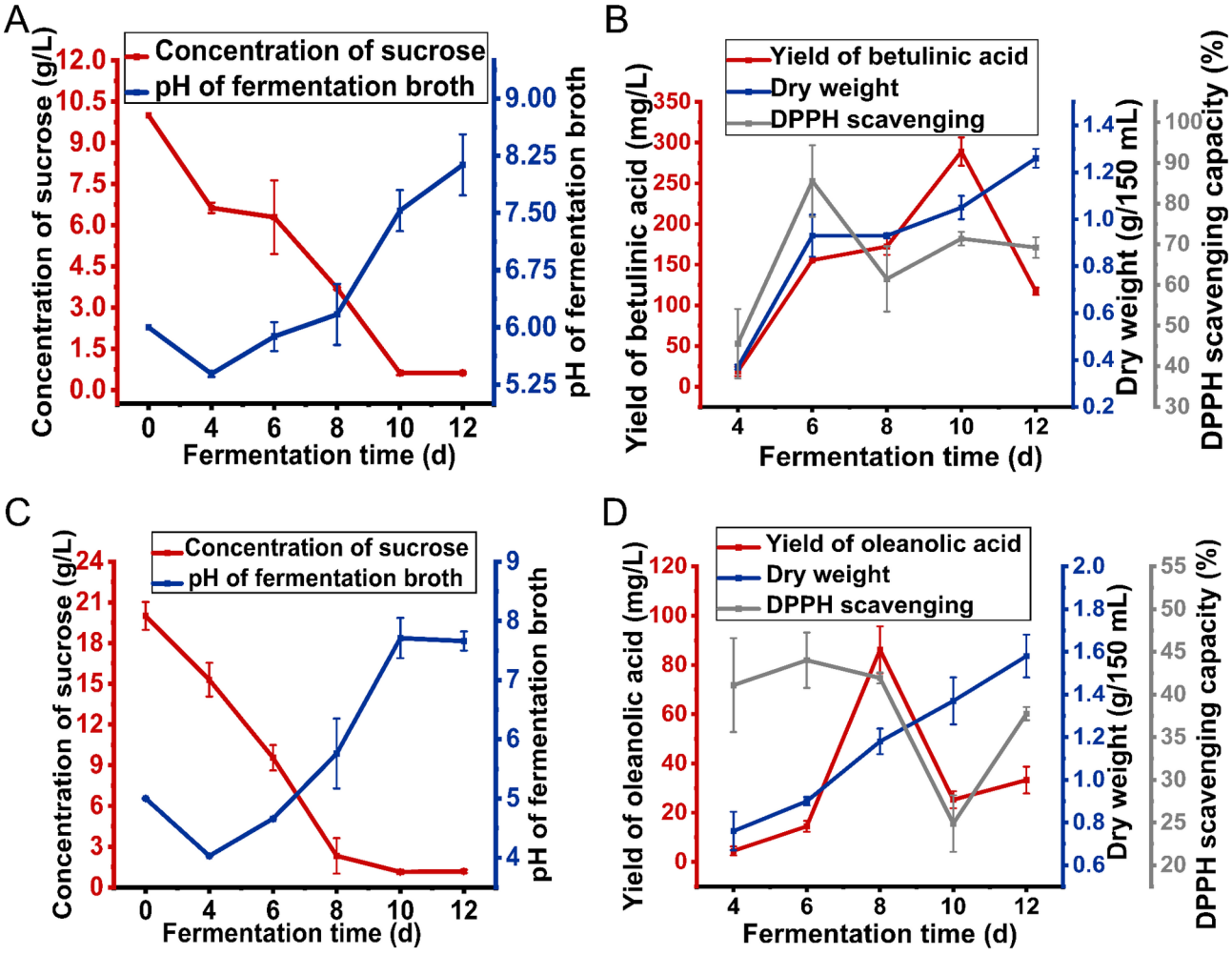


Fig. 4 Effects of fermentation times on growth and triterpene yield of strains. **A**: Combined effects of pH, sucrose, and soybean meal on DPPH scavenging in strain ZBS49; **B**: Effects of incubation time on growth, BA production, and DPPH ability in strain ZBS49; **C**: Combined effects of pH, sucrose, and soybean meal on DPPH scavenging in strain XJ1-1; **D**: Effects of incubation time on growth, OA production, and DPPH ability in strain XJ1-1. Different lowercase letters indicate significant differences ($p < 0.05$)

sucrose, 20 g/L soybean meal extract, and an initial pH of 5 (Fig. 3D, F, H). Based on these optimized medium compositions, we further investigated the optimal cultivation time and dynamic changes occurring during fermentation. Kinetics analysis of fermentation revealed that for strain ZBS49, sucrose consumption was continuous over the first four days, accompanied by a gradual decline in pH (Fig. 4A). By day 5, a significant increase in biomass was observed, coinciding with an acceleration in BA biosynthesis. During this phase, sucrose depletion continued, while pH levels exhibited a steady rebound. Maximum BA production was recorded between days 6 and 10, reaching a peak concentration of 288.97 mg/L on day 10 (Fig. 4B), representing a 5.06-fold increase compared to the pre-optimization yield. As sucrose became fully depleted in the subsequent days (days 11–12), the mycelium transitioned into a senescence phase, leading to a slight decline in BA production. For strain XJ1-1, sucrose was rapidly consumed within the first four days, accompanied by a marked decrease in pH (Fig. 4C). By day 5, the strain entered the logarithmic growth phase, initiating OA biosynthesis. A rapid accumulation of OA was observed between days 6 and 8, during which sucrose levels continued to decline while pH gradually increased (Fig. 4C, D). The maximum OA production of 86.14 mg/L was achieved on day 8, representing a 0.31-fold increase compared to the pre-optimization yield (Fig. 4D). From days 9 to 12, OA production showed an overall downward trend, indicating the onset of the fermentation decline phase.

Additionally, dynamic monitoring of DPPH radical scavenging activity during fermentation provided insights into the antioxidant activity patterns of secondary metabolites. The DPPH scavenging rate of ZBS49 mycelial extract initially increased, followed by a decline, and eventually plateaued, with the peak activity (85.55%)

observed on day 6 (Fig. 4B). This result indicated that the strain generated its highest levels of antioxidant compounds at this stage. By contrast, XJ1-1 exhibited fluctuating DPPH scavenging activity, with an initial slight increase, followed by a decrease and a subsequent increase (Fig. 4D). The peak scavenging rate (44.01%) occurred on day 8, coinciding with the peak OA production, suggesting that OA contributes significantly to the antioxidant activity of XJ1-1.

Inhibition of hepatocellular carcinoma cells by ethanol extract of BA synthetic strain *Clonostachys* sp. ZBS49

BA extract of ZBS49 inhibits the proliferation and migration of liver cancer cells

BA is a potent compound known for its remarkable anticancer properties. Research conducted by Chen et al. demonstrated that BA suppresses GRP78-regulated stemness-related proteins and hinders the polarization into tumor-associated macrophages, thereby diminishing cancer stemness [36]. Additionally, a study by Fernandes et al. emphasized the selective effects of BA against glioblastoma, revealing that it inhibits cell proliferation, induces apoptosis, disrupts the Akt/NFκB-p65 signaling pathway, and enhances the efficacy of temozolomide [37]. To offer significant technical insights and theoretical foundations for using ZBS49 in BA production, paving the way for its application in cancer cell therapy, we conducted the following studies.

In our investigations involving SMMC-7721 and HepG2 cells, we employed Calcein/PI staining and colony formation assays, which showed that treatment with 70 and 140 μM BA significantly reduced cell viability and increased cell mortality (Fig. 5A, B). Additionally, a cell scratch assay was performed to assess the impact of BA on cell migration, revealing a progressive decline in migratory capacity with the administration of 70 and 140 μM BA compared to the control group (Fig. 5C). Collectively, these findings indicate that BA exerts a dose-dependent inhibitory effect on the proliferation and migration of SMMC-7721 and HepG2 cells.

BA extract of ZBS49 induces apoptosis in hepatocellular carcinoma cells

We evaluated the apoptotic effects of the BA extract from ZBS49 on hepatocellular carcinoma cells by treating SMMC-7721 and HepG2 cells with 0, 70, and 140 μM for 24 h. Apoptosis was assessed using the Annexin V-FITC/PI assay, which revealed a significant increase in apoptosis rates. In SMMC-7721 cells, the apoptosis rate rose from 7.58 to 25.9%, while in HepG2 cells, it increased from 4.9 to 30.1% (Fig. 5D). These findings suggest that the BA extract from ZBS49 induces apoptosis in both SMMC-7721 and HepG2 cells in a concentration-dependent manner. Notably, the extract exhibited a half-maximal inhibitory concentration (IC_{50}) of 70 μM, highlighting its potential as a promising anticancer agent.

Genome analysis of *Clonostachys* sp. ZBS49

Assembly and annotation of the genomic sequence of Clonostachys sp. ZBS49

To further elucidate the secondary metabolic pathway of strain ZBS49 and propose potential strategies for optimizing BA production, whole-genome sequencing of strain ZBS49 was performed. The results indicate that the genome size of strain ZBS49 is 57.91 Mb, with a GC content of 49.78%. It contains 18,358 protein-coding genes, with an average coding sequence (CDS) length of 1,770.12 bp. BUSCO analysis confirmed the high completeness of the genome assembly, with a completeness score of 99.3%, indicating its exceptional quality. Non-coding RNA prediction revealed the presence of 236 tRNA genes and 49 rRNA genes. Additionally, the genome contains 58 short dispersed repeats, 251 long dispersed repeats, 4 long terminal repeats, 68 DNA transposons (accounting for 0.9% of the genome), 5,401 simple sequence repeats, and 3 satellite repeats. The functional annotation of the COG identified 8,137 genes with undefined functions. Among these, 1,255 genes were associated with carbohydrate transport and metabolism. GO annotation correlated 3,268 genes to metabolic processes, 6,323 genes to catalytic activity, and 4,929 genes to binding functions. KEGG analysis indicated that 579 genes are implicated in carbohydrate metabolism, while 346 are related to transport and catabolic pathways (Fig. 6). The high expression of genes in related metabolic pathways, especially those related to secondary metabolism, provides an essential basis and reference for subsequent secondary metabolism research.

Phylogenetic analysis of Clonostachys sp. ZBS49

To analyze the phylogenetic of the endophytic fungus ZBS49, we selected 11 fungal species from the same genus and relatively close relatives within the order *Carnobacteria* for a single-copy direct homologous sequence analysis. OrthoFinder identified 162,137 genes (93.8%) across 17,415 gene families for ZBS49 and the 11 other fungal species involved. In total, 4,309 gene families were recognized across all species, of which 3,232 were composed entirely of single-copy genes. The results indicated that ZBS49, compared to the other 11 reference fungi, possessed 17,394 genes categorized into 13,621 gene families and 1,030 unassigned gene families. We employed the STRIDE algorithm to construct a phylogenetic tree for ZBS49 and the 11 other fungal species within the order *Carnobacteria*, analyzing the taxonomic status of ZBS49 and delineating the homologous relationships among various genera based on the highly conserved single-copy direct homologous sequences. The analysis

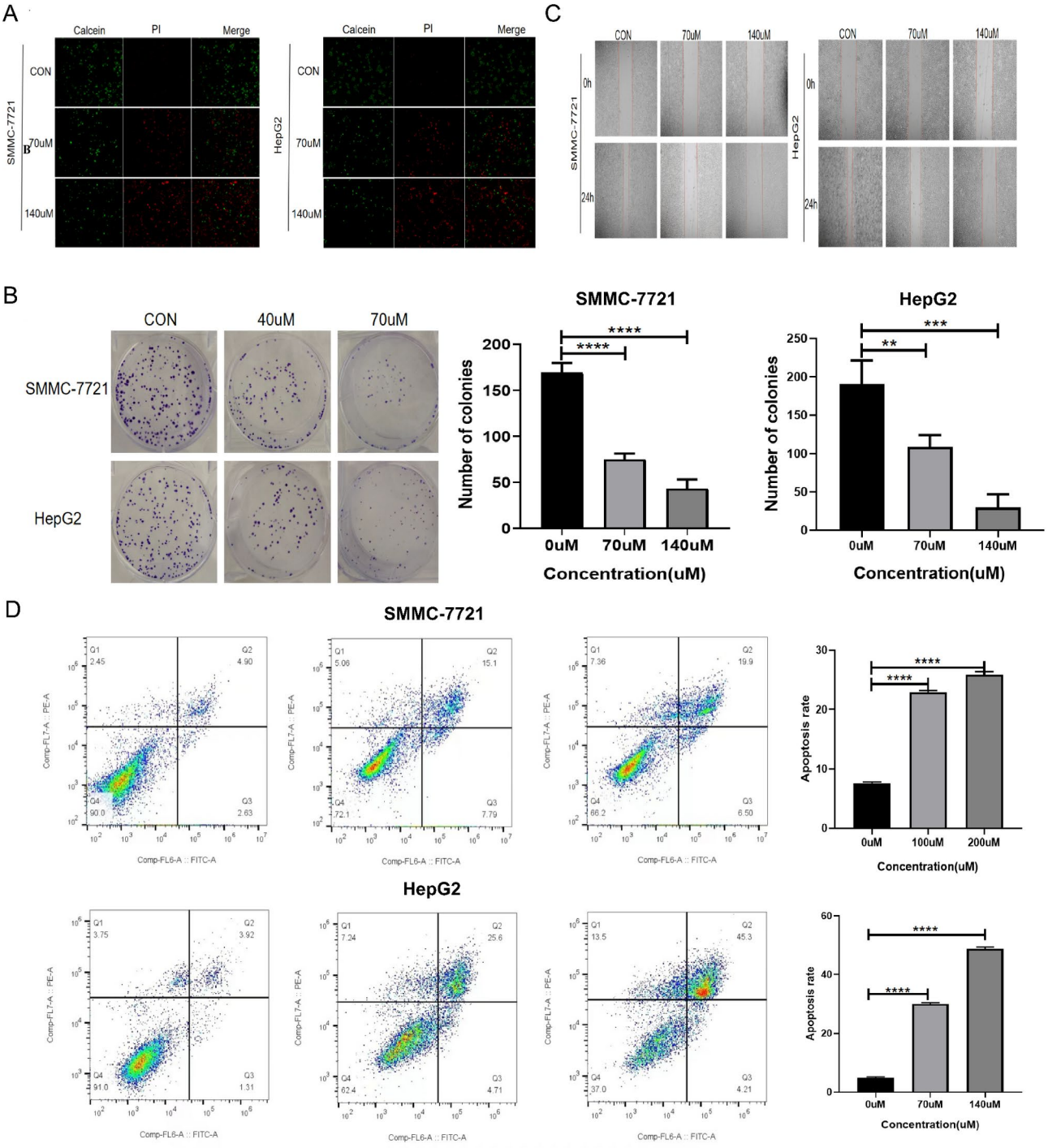


Fig. 5 BA extract of ZBS49 inhibited the proliferation and migration of hepatocellular carcinoma cells. Note: **A**: Calcein/PI staining of SMMC-7721 and HepG2 cells following 24 h of incubation with BA (0 μM, 70 μM, 140 μM) or an equivalent volume of DMEM medium. **B**: SMMC-7721 and HepG2 cells incubated with BA (0 μM, 70 μM, 140 μM) or an equal volume of DMEM medium for 24 h, showcasing a clone formation assay. **C**: SMMC-7721 and HepG2 cells underwent a wound healing assay after incubation with BA (0 μM, 70 μM, 140 μM) or an equal volume of DMEM medium for 24 h. **D**: The apoptosis rate of SMMC-7721 and HepG2 cells was assessed as (Q2 + Q3)/(Q1 + Q2 + Q3 + Q4) through flow cytometry, with the cells incubated with BA (0 μM, 70 μM, and 140 μM) or an equivalent volume of DMEM medium for 24 h before the assay. *Data are mean ± SD, $N = 3$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

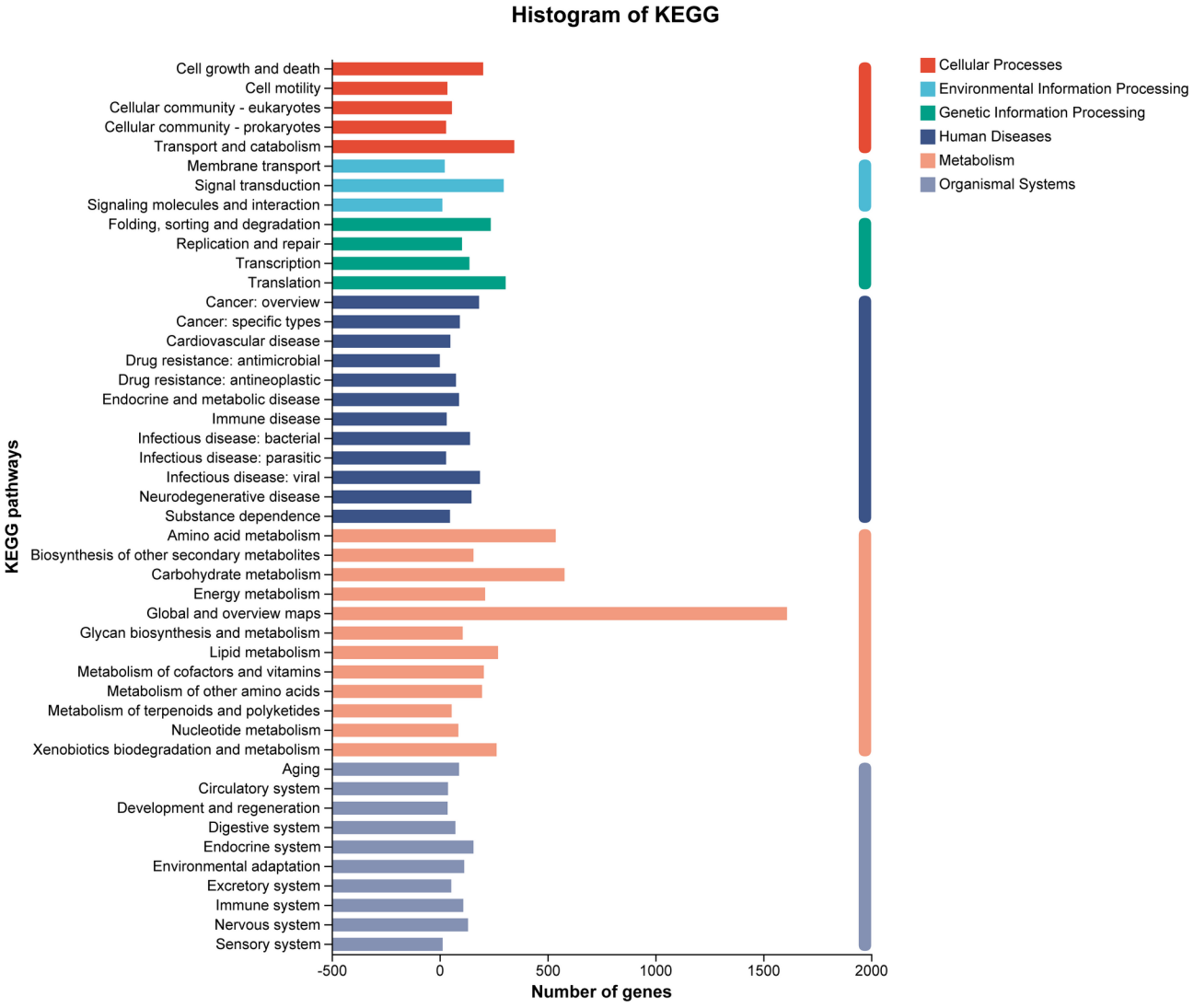


Fig. 6 KEGG Cluster Analysis of ZBS49 Genomic Sequence

revealed that ZBS49 is the closest relative to *Clonostachys rosea* f. *rosea*. Based on genome phylogenetic analyses, ZBS49 belongs to the *Sarcoidales* order, *Chlorophoraceae* family, and *Clonostachys* genus (Fig. 7).

Elucidation of the biosynthetic pathway of BA in *Clonostachys* sp. ZBS49

Elucidation of the terpene precursor pathway genes in ZBS49

To further explore the BA biosynthesis pathway of *Clonostachys* sp. ZBS49, 16 key precursor genes involved in BA biosynthesis, were identified from the genome. The enzymes encoded by these genes include 2 acetyl-CoA C-acetyltransferases, 1 hydroxymethylglutaryl-CoA synthase, 1 hydroxymethylglutaryl-CoA reductase, 2 mevalonate kinases, 1 phosphomevalonat kinase, 1 diphosphomevalonate decarboxylase, 1 isopentenyl-di phosphate delta-isomerase, 1 geranylgeranyl diphosphate synthase, 2 farnesyl diphosphate synthases, 1 squalene synthase, 1 squalene monooxygenase, and 2 squalene-hopene cyclases (Fig. 8A). Genomic localization analysis indicated that these genes are predominantly found on scaffolds 4 and 14 (Fig. 8B). Furthermore, through AntiSMASH fungal 7.0.0 analysis, six terpene biosynthetic gene clusters were identified in ZBS49 (Fig. 8C). Among them, cluster 1 and cluster 4 have been confirmed as triterpene biosynthetic gene clusters, containing coding genes for enzymes such as CYP450, dioxygenase, and glycosyltransferase.

Analysis of the CYP450 family in the ZBS49 genome and screening for CYP450 genes with C-28 oxidation activity

To provide critical insights into the biosynthetic pathways of BA and establish a theoretical framework for further investigation into the C-28 oxidation functions of the CYP450 gene family in fungi, we isolated 145 complete CYP450 genes following rigorous filtering

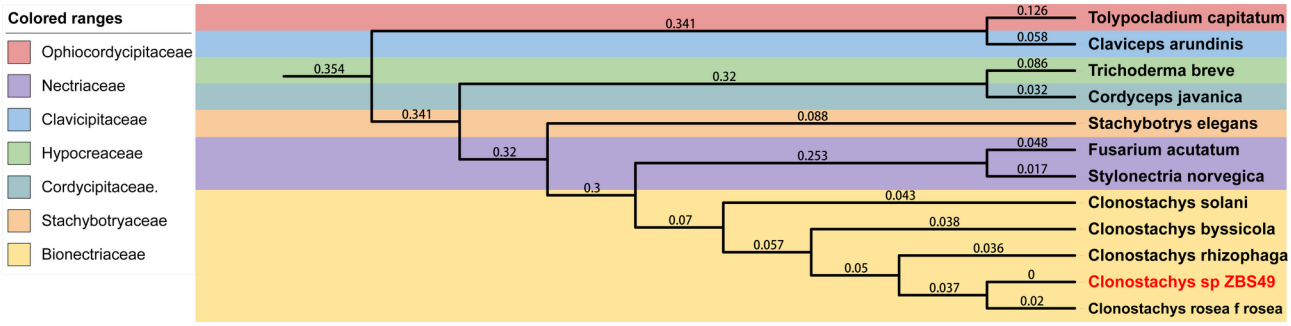


Fig. 7 Phylogenetic tree analysis of *Clonostachys* sp

and selection processes. Phylogenetic clustering analysis (Fig. 9) revealed that 116 of the identified CYP450 genes were classified into eight distinct clades: clade 1 (2 genes), clade 2 (5 genes), clade 3 (3 genes), clade 8 (65 genes), clade 9 (2 genes), clade 10 (19 genes), clade 14 (2 genes), and clade 15 (18 genes). Further analysis of the motifs and conserved domains within the CYP450 genes from the ZBS49 genome indicated that motifs 1 and 2 are highly conserved and prevalent across the majority of these genes, suggesting that these motifs may represent unique conserved features characteristic of the ZBS49 CYP450 family.

These enzymes are involved in the oxidation or hydroxylation of specific sites during the biosynthesis of various triterpenoid compounds. Notably, CYP450 genes exhibiting C-28 oxidation activity are predominantly found within the plant Steroid 2-Cysteine family, which are known to catalyze the oxidation of α- and β-amyrins and lupeol [38, 39]. To investigate the presence of analogous functional CYP450 genes in fungi, we performed a clustering analysis juxtaposing known plant CYP450 genes with those identified in the ZBS49 genome. The study revealed that five genes (g04998, g08573, g14573, g10613, g14162) clustered with plant C-28 oxidation CYP450 genes within the same evolutionary branch and were classified into the Steroid 2-Cysteine family *CYP120A*.

Discussion

Triterpenoid compounds such as BA and OA have significant biological activities, including hepatoprotective, anti-inflammatory, antiviral, and anticancer effects, making them highly valuable in the pharmaceutical and healthcare fields [40]. However, the current production of these compounds primarily relies on plant extraction and chemical synthesis, which suffer from low efficiency, complex extraction processes, and environmental concerns [41]. Consequently, developing sustainable biosynthetic approaches has become a key research focus. Through long-term co-evolution, endophytic fungi have acquired the ability to synthesize metabolites identical or similar to those of their host plants, positioning them as a promising natural source for producing bioactive compounds [15, 16]. In recent years, researchers have isolated the initial strain *Epicoccum nigrum* TXB502 from *Taxus*, which is capable of synthesizing paclitaxel in natural environments. Through process optimization and immobilization techniques, the paclitaxel concentration was increased approximately 22-fold [42]. Previous studies have demonstrated that endophytic fungi possess the ability to synthesize BA and OA. Peyrat et al. isolated an endophytic fungus with significant antibacterial activity from *Thuja Sutchuenensis*, identifying 10 terpenoid compounds in its fermentation products, including BA and OA. However, the low yield of these compounds poses a challenge to industrial-scale production [27]. To address this issue, we systematically screened 171 endophytic fungal strains isolated from various triterpene-producing plants. As a result, we successfully identified 43 strains capable of converting BT to BA, 43 strains capable of synthesizing BA, and 41 strains capable of synthesizing OA. Without genetic modification, strain ZBS49 achieved a BA yield of 47.7 mg/L, and strain XJ1-1 produced OA at a yield of 65.76 mg/L, representing the highest production levels reported to date among naturally occurring filamentous fungi. Filamentous fungi possess a robust protein secretion system and a comprehensive post-translational modification mechanism, enabling efficient heterologous protein expression while ensuring correct eukaryotic protein folding. Additionally, their well-established submerged fermentation technology reduces industrial production costs. These advantages collectively position filamentous fungi as an essential group of industrial microorganisms with widespread applications across multiple sectors, including food, feed, biofuels, biochemical products, pharmaceuticals, paper production, detergents, textiles, waste management, and agriculture [43, 44]. Filamentous fungi widely utilized for large-scale industrial production of natural products primarily include *Aspergillus*, *Trichoderma*, *Penicillium*, and *Myceliophthora*, with *Fusarium*, *Rhizopus*, and *Mucor* being applied to a lesser extent. These fungal species are predominantly employed in producing cephalosporin

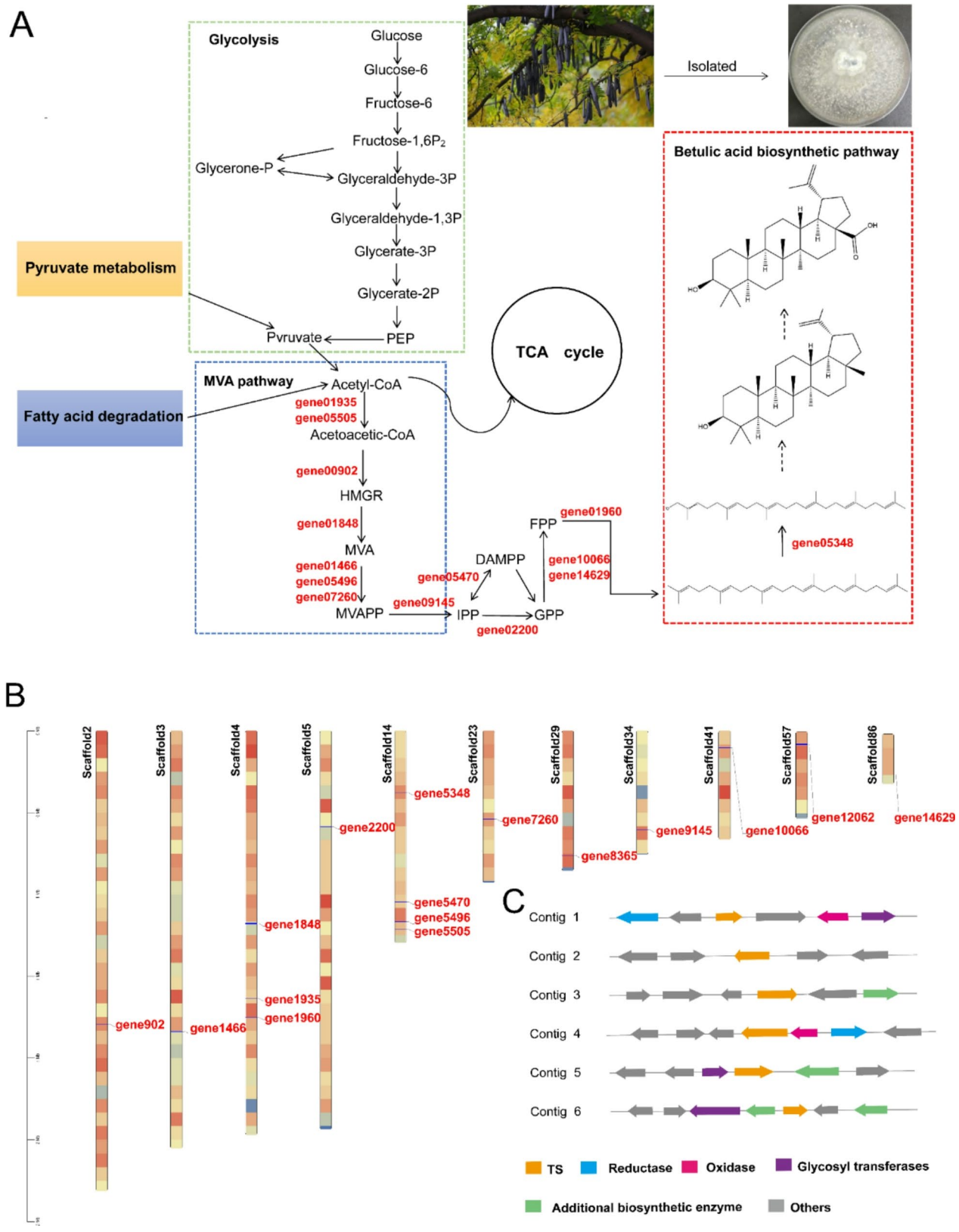


Fig. 8 Analysis of terpenoid-related genes in *Clonostachys* sp. Note: **A**: Genes associated with predicted terpenoid synthesis pathway in strain ZBS49; **B**: Genomic localization of terpenoid-related genes; **C**: Terpenoid biosynthetic gene clusters analysis in strain ZBS49

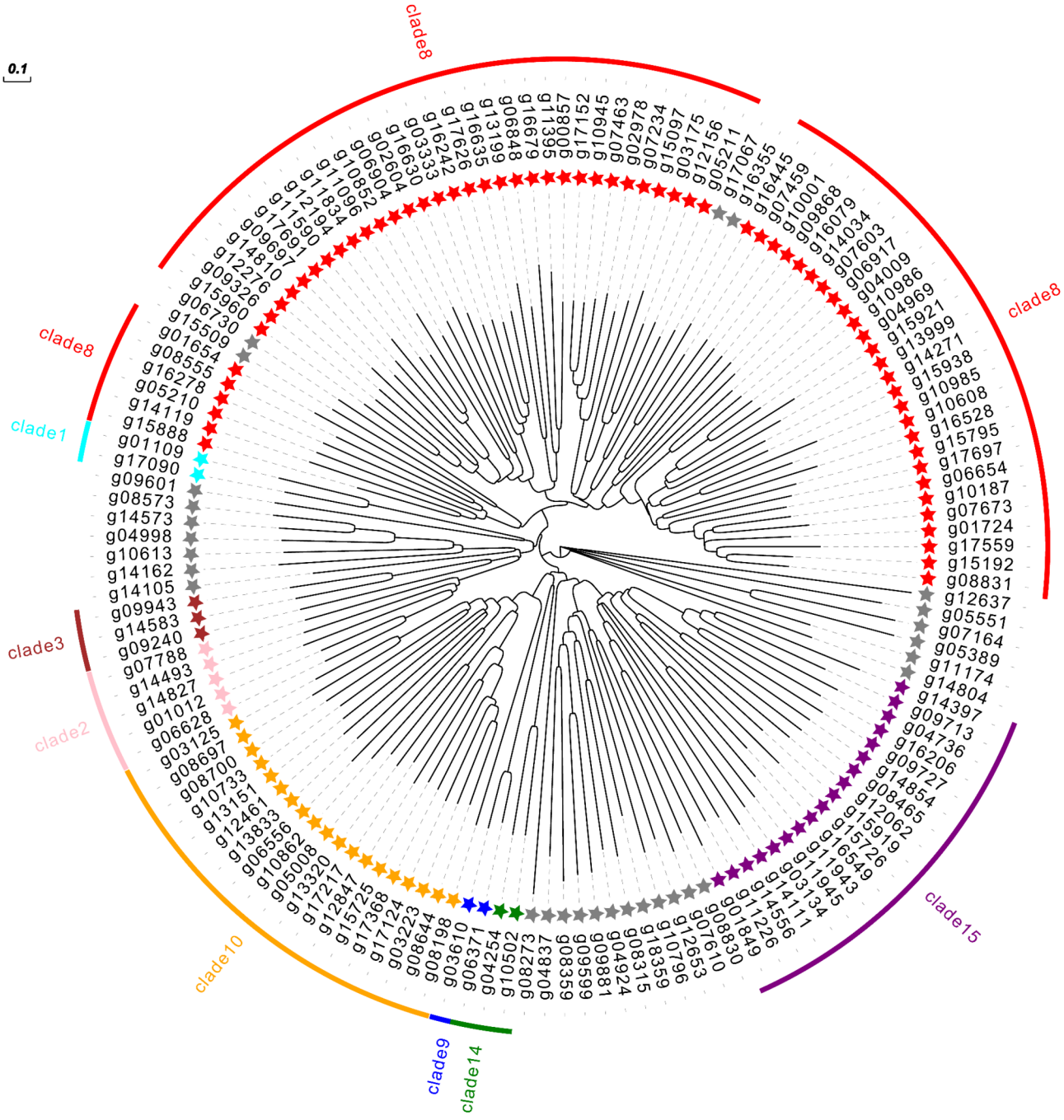


Fig. 9 CYP450 phylogenetic tree of ZBS49 genome

C, statins, cellulases, and organic acids [45–49]. In this study, we have first identified a fungal strain capable of efficiently synthesizing BA and OA. This discovery establishes a crucial strain foundation for the industrial production of BA and OA, holding significant theoretical implications and practical applications.

The growth and development of microbial mycelia and the biosynthesis of secondary metabolites are significantly influenced by the type of culture medium, nutrient composition, and cultivation conditions. Studies have demonstrated that optimizing fermentation parameters can substantially enhance the yield of target products. For instance, Sun et al. systematically optimized the culture conditions of *Tremella aurantialba* NX-20, increasing the yield of the target product to 15.02 ± 0.40 g/L [50]. Similarly, Qiu et al. improved the fermentation process of *Aspergillus favipes* HN4-13, achieving an emodin yield of 132.40 ± 3.09 mg/L [51]. Previous studies have

demonstrated that the biosynthesis of xylitol is regulated mainly by fermentation parameters such as cultivation temperature, pH, agitation speed, and concentrations of carbon and nitrogen sources [52]. Furthermore, the efficiency of erythritol synthesis is also significantly influenced by factors including the type of carbon and nitrogen sources, pH, and temperature [53]. Based on these findings, this study systematically investigated the effects of 6 different culture media on the growth of the strain and the biosynthesis of its metabolic products. The results demonstrated that the soybean meal medium significantly promoted both strain growth and triterpenoid biosynthesis, leading to a 192% increase in the production of BA by strain ZBS49 compared to the PDB medium. This enhancement can be attributed to two potential factors: first, the soybean meal medium is rich in secondary metabolite precursors such as squalene [54], providing a sufficient substrate supply for triterpenoid biosynthesis in strains ZBS49 and XJ1-1; second, soybean meal, as an organic nitrogen source with a high protein content (44%), possesses a well-balanced composition of essential amino acids, which confers significant advantages in microbial fermentation. Previous studies have demonstrated the successful application of soybean meal in the biosynthesis of lactic acid and surfactin [55, 56]. Building upon this foundation, this study further conducted single-factor optimization of the soybean-based medium. The results demonstrated that nitrogen source, carbon source concentration, and pH significantly influenced the growth of strains ZBS49 and XJ1-1, as well as the biosynthesis of BA and OA. Precisely, when sucrose and soybean meal extract were added at concentrations of 10 g/L, and the pH was adjusted to 6, strain ZBS49 achieved its highest BA yield of 288.97 mg/L on the 10th day of fermentation, representing a 506%-fold increase than that before optimization. For strain XJ1-1, the highest yield of OA, 86.14 mg/L, was obtained on the 8th day of fermentation when sucrose and soybean meal extract were added at concentrations of 20 g/L and the pH was adjusted to 5, representing a 31%-fold increase compared to pre-optimization conditions. To date, studies on the optimization of culture conditions for BA and OA biosynthesis by filamentous fungi remain scarce. This study represents the first application of a soybean-based medium to produce BA and OA. The optimized fermentation system significantly enhanced triterpenoid yields, providing important theoretical and practical insights for the cost-effective biosynthesis of triterpenoid compounds using filamentous fungi.

Transcriptomics and genomics-based approaches have become integral strategies for investigating the natural products of various organisms [57, 58]. These methodologies enable the identification of potential biosynthetic pathways for secondary metabolites isolated from organisms, activating silent pathways and the rational design of new molecules, thus promoting the biosynthesis of valuable secondary metabolites. Compared to traditional natural product discovery methods, genome-guided natural product mining allows for more precise identification of the secondary metabolites encoded by the strains, thus accelerating the discovery of novel bioactive natural products. Although fungal genomes typically contain a wealth of genes involved in secondary metabolite biosynthesis, most of these genes are either not expressed or only weakly expressed under standard laboratory culture conditions, necessitating further exploration. For example, Yang et al. used the protein sequence of the sesquiterpene cyclase *EriG* as a probe for genomic mining, successfully identified 7 new terpene cyclases, further elucidating their product structures, including the novel sesquiterpene scaffold lydicene [59]. Similarly, Chen et al. performed whole-genome sequencing of the basidiomycete *Irpex lacteus*, identified the tremulane sesquiterpene synthase, and heterologously expressed it in *Aspergillus oryzae*, leading to the production of four new sesquiterpenes Iltremulanols A-D [60]. In this study, an ITS sequence alignment revealed that strain ZBS49 shares 99.82% sequence similarity with *Clonostachys* species, suggesting it may represent a new strain within this genus. Genome sequencing of ZBS49 revealed a genome size of 57.91 Mb, consistent with previously reported genome sizes for fungi within the same genus [61, 62]. To date, no studies have reported the triterpene biosynthetic pathway of the genus *Clonostachys*. However, given its potential in BA synthesis, it is particularly important to thoroughly investigate the genes involved in its efficient BA production. The biosynthesis of fungal terpenoid natural products begins with the MVA pathway, which synthesizes the two five-carbon intermediates, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These intermediates are then catalyzed by FPP synthase (FPPS), squalene synthase (SQS), and squalene epoxidase (SQE) to form 2,3-oxidosqualene [63]. Furthermore, Tian-Gang Liu's team discovered a unique triterpene biosynthesis pathway in fungi independent of the squalene synthesis route. In this pathway, the isoprenyl transferase domain of the Type I chimeric terpene cyclases *TvTS* and *MpMS* catalyzes the condensation of IPP and DMAPP to form hexaprenyl pyrophosphate (HexPP), which is then cyclized by the terpene cyclase domain to produce a novel triterpene scaffold [64]. In this study, genomic analysis of strain ZBS49 identified 16 genes related to triterpene biosynthesis, along with two triterpene biosynthetic gene clusters containing CYP450, dioxygenases, and glycosyltransferases. This study represents the first systematic analysis of the triterpene biosynthetic pathway in *Clonostachys* sp., providing a foundation for understanding the metabolic potential of

strain ZBS49 and offering new directions for optimizing BA production.

CYP450 enzymes play a crucial role in triterpene biosynthesis, such as catalyzing oxidation at various positions on the carbon ring backbone to form unsaturated groups like hydroxyl, carboxyl, ketone, and double bonds [65]. To date, only a limited number of CYP450 genes have been reported to be involved in the modification of triterpenoid scaffolds in filamentous fungi, such as *HelB1-HelB4*, *FusB1*, *CepB4*, *CYP512U6* and *AfumB*, which catalyze oxidation or hydroxylation of specific sites during the biosynthesis of various triterpenes [66–70]. The CYP450 genes involved in the synthesis of BA mainly belong to the plant CYP716 family. For example, *RoCYP716A155* from *Rosmarinus officinalis* effectively converts lupeol to BA [39]. Similarly, *CYP716A12* from *Medicago truncatula* can catalyze a three-step oxidation of β-amyrin and lupeol to produce ursolic acid (UA) and BA, respectively [38]. *CYP716A179* from *Glycyrrhiza uralensis*, expressed heterologously in engineered yeast strains, was shown to catalyze the conversion of β-amyrin, α-amyrin, and lupeol into UA, OA, and BA, respectively [71]. In *Arabidopsis thaliana*, CYP716A1 and CYP716A2 catalyze the hydroxylation of lupeol to form BA through a single hydroxylation reaction [72]. To explore similar CYP450 genes in strain ZBS49, this study performed a clustering analysis of known plant CYP450 genes and P450 genes in the ZBS49 genome. The results revealed that 5 genes (g04998, g08573, g14573, g10613, g14162) belong to the same evolutionary branch as known plant C-28 oxidizing CYP450 genes and are all classified under the CYP120A family. This finding provides a significant theoretical foundation for further in-depth investigation of the biosynthetic pathway of BA, while also laying the groundwork for research on the P450 gene family responsible for C-28 oxidation in fungi.

Conclusion

This study presented the first report on two endophytic fungal strains, ZBS49 and XJ1-1, recognized for their remarkable natural production of BA (47.7 mg/L) and OA (65.76 mg/L). ITS sequence analysis revealed that ZBS49 shares 99.82% similarity with known strains, suggesting that ZBS49 may be classified as a novel species within the genus *Clonostachys*. Optimization of culture media and growth conditions resulted in significant enhancements in BA and OA production, achieving yields of 288.97 and 86.14 mg/L, which reflect increases of 506% and 31%, respectively. The BA extract derived from ZBS49 exhibited a significant dose-dependent inhibitory effect on hepatocellular carcinoma (HCC) cell lines, specifically SMMC-7721 and HepG2. Notably, the extract showed an IC_{50} of 70 μM, suggesting its potential utility as an anticancer agent. The biosynthetic pathway of BA in ZBS49 has been elucidated for the first time, revealing the involvement of 16 genes in triterpenoid biosynthesis alongside 2 triterpene biosynthetic gene clusters and 5 CYP450 genes potentially associated with C-28 oxidation. We establish a robust technical and theoretical foundation for utilizing ZBS49 as a microbial chassis for triterpenoid biosynthesis, highlighting its potential applications in cancer therapy. Furthermore, we emphasize the viability of employing filamentous fungi as biofactories to efficiently produce BA and its derivatives, providing valuable genetic resources for metabolic engineering and biotechnological advancements.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02733-w.

Supplementary Material 1

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Author contributions

LG and JC: Methodology, Validation, Investigation, Writing - original draft. CX and LX: Validation, Formal analysis, Data curation. FY and TZ: Validation, Formal analysis. MZ: Software, Data curation. ZL and YR: Software. CW: Resources, Project administration. JY: Conceptualization, Funding acquisition, Project administration, Supervision, Writing review & editing.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Morparia S, Metha C, Suvarna V. Recent advancements of betulinic acid-based drug delivery systems for cancer therapy (2002–2023). Nat Prod Res. 2024;1–21.
2. Ren J, Yan J, Raza F, Zafar H, Wan H, Chen X, Cui Q, Li H, Wang X. A synergistic combination of oleanolic acid and apatinib to enhance antitumor effect on

liver Cancer cells and protect against hepatic injury. Recent Pat Anticancer Drug Discov. 2024;19(2):199–208.
3. Wang K, Shang J, Tao C, Huang M, Wei D, Yang L, Yang J, Fan Q, Ding Q, Zhou M. Advancements in betulinic Acid-Loaded nanoformulations for enhanced Anti-Tumor therapy. Int J Nanomed. 2024;19:14075–103.
4. Zeng LR, Pan BW, Cai J, Liu LJ, Dong ZC, Zhou Y, Feng TT, Shi Y. Construction, structural modification, and bioactivity evaluation of pentacyclic triterpenoid privileged scaffolds in active natural products. RSC Adv. 2024;14(53):39436–61.
5. Zhang S, Peng B, Chen Z, Yu J, Deng G, Bao Y, Ma C, Du F, Sheu WC, Kimberly WT, et al. Brain-targeting, acid-responsive antioxidant nanoparticles for stroke treatment and drug delivery. Bioact Mater. 2022;16:57–65.
6. Zhou Y, Wang H, Zhu X, Zhao Q, Deng G, Li Y, Chen Q. Improving anti-oxidant stress treatment of subarachnoid hemorrhage through self-assembled nanoparticles of oleanolic acid. Drug Deliv. 2024;31(1):2388735.
7. Liu J, Lu YF, Wu Q, Xu SF, Shi FG, Klaassen CD. Oleanolic acid reprograms the liver to protect against hepatotoxicants, but is hepatotoxic at high doses. Liver Int. 2019;39(3):427–39.
8. Lugiņina J, Kroškins V, Lācis R, Fedorovska E, Demir Ö, Dubnika A, Loca D, Turks M. Synthesis and preliminary cytotoxicity evaluation of water soluble pentacyclic triterpenoid phosphonates. Sci Rep. 2024;14(1):28031.
9. Fonseca FCS, Reis LCB, Dos Santos JDG, Branco CRC, Ferreira S, David JM, Branco A. Betulinic acid from Zizyphus Joazeiro bark using focused Microwave-Assisted extraction and response surface methodology. Pharmacogn Mag. 2017;13(50):226–9.
10. Medina E, Romero C, Brenes M. Residual Olive paste as a source of phenolic compounds and triterpenic acids. Eur J Lipid Sci Technol. 2018;120(4):1700368.
11. Li Y, Wang J, Li L, Song W, Li M, Hua X, Wang Y, Yuan J, Xue Z. Natural products of pentacyclic triterpenoids: from discovery to heterologous biosynthesis. Nat Prod Rep. 2023;40(8):1303–53.
12. Ma YR, Wang KF, Wang WJ, Ding Y, Shi TQ, Huang H, Ji XJ. Advances in the metabolic engineering of *Yarrowia lipolytica* for the production of terpenoids. Bioresour Technol. 2019;281:449–56.
13. Qin X, Xu J, An X, Yang J, Wang Y, Dou M, Wang M, Huang J, Fu Y. Insight of endophytic fungi promoting the growth and development of Woody plants. Crit Rev Biotechnol. 2024;44(1):78–99.
14. Woo SL, Hermosa R, Lorito M, Monte E. *Trichoderma*: a multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. Nat Rev Microbiol. 2023;21(5):312–26.
15. Chen Y, Hu B, Xing J, Li C. Endophytes: the novel sources for plant terpenoid biosynthesis. Appl Microbiol Biotechnol. 2021;105(11):4501–13.
16. Khattab AR, Farag MA. Marine and terrestrial endophytic fungi: a mine of bioactive Xanthone compounds, recent progress, limitations, and novel applications. Crit Rev Biotechnol. 2022;42(3):403–30.
17. Cao X, Xu L, Wang J, Dong M, Xu C, Kai G, Wan W, Jiang J. Endophytic fungus *Pseudodidymocyrtis lobariellae* KL27 promotes taxol biosynthesis and accumulation in Taxus chinensis. BMC Plant Biol. 2022;22(1):12.
18. Huang JX, Zhang J, Zhang XR, Zhang K, Zhang X, He XR. *Mucor fragilis* as a novel source of the key pharmaceutical agents Podophyllotoxin and Kaempferol. Pharm Biol. 2014;52(10):1237–43.
19. Sang X, Yang M, Su J. Research on endophytic fungi for producing huperzine A on a large-scale. Crit Rev Microbiol. 2020;46(6):654–64.
20. Li N, Xu D, Huang RH, Zheng JY, Liu YY, Hu BS, Gu YQ, Du Q. A new source of diterpene lactones from *Andrographis paniculata* (Burm. f.) Nees-Two endophytic Fungi of *Colletotrichum* Sp. With antibacterial and antioxidant activities. Front Microbiol. 2022;13:819770.
21. Rajani P, Rajasekaran C, Vasanthakumari MM, Olsson SB, Ravikanth G, Uma Shaanker R. Inhibition of plant pathogenic fungi by endophytic *Trichoderma* spp. Through mycoparasitism and volatile organic compounds. Microbiol Res. 2021;242:126595.
22. Shah S, Shah B, Sharma R, Rekadwad B, Shouche YS, Sharma J, Pant B. Colonization with non-mycorrhizal culturable endophytic fungi enhances Orchid growth and Indole acetic acid production. BMC Microbiol. 2022;22(1):101.
23. Zhu M, He C, Zhou J, Li Y, Qian L, Zhang J, Qiao Y, Chang W, Lou H. Liverwort-Derived metabolites retard endophyte growth and inspire antifungal application. J Agric Food Chem. 2023;71(12):4863–75.
24. Chen X, Shen W, Chen J, Zhu Y, Chen C, Xie S. Tetracycline biotransformation by a novel bacterial strain *Alcaligenes* Sp. T17. Sci Total Environ. 2022;832:155130.
25. Tang R, Shen Y, Xia M, Tu L, Luo J, Geng Y, Gao T, Zhou H, Zhao Y, Wang M. A highly efficient step-wise biotransformation strategy for direct conversion of phytosterol to boldenone. Bioresour Technol. 2019;283:242–50.
26. Yang SB, Zheng HC, Xu JY, Zhao XY, Shu WJ, Li XM, Song H, Ma YH. New biotransformation mode of Zearalenone identified in *Bacillus subtilis* Y816 revealing a novel ZEN conjugate. J Agric Food Chem. 2021;69(26):7409–19.
27. Peyrat LA, Eparvier V, Eydoux C, Guillemot JC, Litaudon M, Stien D. Betulinic acid, the first lupane-type triterpenoid isolated from both a *Phomopsis* Sp. and its host plant diospyros carbonaria benoist. Chem Biodivers. 2017;14(1):10. https://doi.org/10.1002/cbdv.201600171
28. Feng Y, Li M, Liu J, Xu TY, Fang RS, Chen QH, He GQ. A novel one-step microbial transformation of betulin to betulinic acid catalysed by *Cunninghamella Blakesleeana*. Food Chem. 2013;136(1):73–9.
29. Chen HJ, Chen YS, Liu SL, Liou BK, Chen CS. The influence of submerged fermentation of inonotus obliquus with control atmosphere treatment on enhancing bioactive ingredient contents. Appl Biochem Biotechnol. 2020;191(1):412–25.
30. Lou H, Li H, Wei T, Chen Q. Stimulatory effects of oleci acid and fungal elicitor on betulinic acid production by submerged cultivation of medicinal mushroom *Inonotus obliquus*. J Fungi (Basel). 2021;7(4):1725.
31. Liu SJ, Liu Z, Shao BY, Li T, Zhu X, Wang R, Shi L, Xu S, Van de Peer Y, Xue JY. Deciphering the biosynthetic pathway of triterpene saponins in *Prunella vulgaris*. Plant J. 2025;121(2):e17220.
32. Tang M, Xu X, Liu Y, Li J, Du G, Lv X, Liu L. Combinatorial metabolic engineering for improving betulinic acid biosynthesis in *Saccharomyces cerevisiae*. ACS Synth Biol. 2024;13(6):1798–808.
33. Cheng X, Pang Y, Ban Y, Cui S, Shu T, Lv B, Li C. Application of multiple strategies to enhance oleanolic acid biosynthesis by engineered *Saccharomyces cerevisiae*. Bioresour Technol. 2024;401:130716.
34. Yin J, Li X, Zhan Y, Li Y, Qu Z, Sun L, Wang S, Yang J, Xiao J. Cloning and expression of BpMYC4 and BpbHLH9 genes and the role of BpbHLH9 in triterpenoid synthesis in Birch. BMC Plant Biol. 2017;17(1):214.
35. Yin JL, Wong WS, Jang IC, Chua NH. Co-expression of peppermint Geranyl diphosphate synthase small subunit enhances monoterpene production in Transgenic tobacco plants. New Phytol. 2017;213(3):1133–44.
36. Chen JL, Tai YS, Tsai HY, Hsieh CY, Chen CL, Liu CJ, et al. Betulinic acid inhibits the stemness of gastric cancer cells by regulating the GRP78-TGF-β1 signaling pathway and macrophage polarization. Molecules. 2023;28(4):1725.
37. Fernandes S, Vieira M, Prudêncio C, Ferraz R. Betulinic acid for glioblastoma treatment: reality, challenges and perspectives. Int J Mol Sci. 2024;25(4):2108.
38. Fukushima EO, Seki H, Ohyama K, Ono E, Umemoto N, Mizutani M, Saito K, Muranaka T. CYP716A subfamily members are multifunctional oxidases in triterpenoid biosynthesis. Plant Cell Physiol. 2011;52(12):2050–61.
39. Huang J, Zha W, An T, Dong H, Huang Y, Wang D, Yu R, Duan L, Zhang X, Peters RJ, et al. Identification of *RoCYP01* (CYP716A155) enables construction of engineered yeast for high-yield production of betulinic acid. Appl Microbiol Biotechnol. 2019;103(17):7029–39.
40. Luo Y, Jiang Y, Chen L, Li C, Wang Y. Applications of protein engineering in the microbial synthesis of plant triterpenoids. Synth Syst Biotechnol. 2023;8(1):20–32.
41. Guo M, Lv H, Chen H, Dong S, Zhang J, Liu W, He L, Ma Y, Yu H, Chen S, Luo H. Strategies on biosynthesis and production of bioactive compounds in medicinal plants. Chin Herb Med. 2024;16(1):13–26.
42. El-Sayed ER, Zaki AG, Ahmed AS, Ismaiel AA. Production of the anticancer drug taxol by the endophytic fungus *Epicoccum nigrum* TXB502: enhanced production by gamma irradiation mutagenesis and immobilization technique. Appl Microbiol Biotechnol. 2020;104(16):6991–7003.
43. Kalra R, Conlan XA, Goel M. Fungi as a potential source of pigments: Harnessing filamentous Fungi. Front Chem. 2020;8:369.
44. Madhavan A, Arun KB, Sindhu R, Alphonsa Jose A, Pugazhendhi A, Binod P, et al. Engineering interventions in industrial filamentous fungal cell factories for biomass valorization. Bioresour Technol. 2022;344(Pt A):126209.
45. Dai Z, Guo F, Zhang S, Zhang W, Yang Q, Dong W, Jiang M, Ma J, Xin F. Bio-based succinic acid: an overview of strain development, substrate utilization, and downstream purification. Biofuels Bioprod Biorefin. 2020;14(5):965–85.
46. Guo F, Wu M, Dai Z, Zhang S, Zhang W, Dong W, Zhou J, Jiang M, Xin F. Current advances on biological production of fumaric acid. Biochem Eng J. 2020;153:107397.
47. Liu L, Chen Z, Liu W, Ke X, Tian X, Chu J. Cephalosporin C biosynthesis and fermentation in *Acremonium chrysogenum*. Appl Microbiol Biotechnol. 2022;106(19–20):6413–26.

48. Meyer V, Basenko EY, Benz JP, Braus GH, Caddick MX, Csukai M, de Vries RP, Endy D, Frisvad JC, Gunde-Cimerman N, et al. Growing a circular economy with fungal biotechnology: a white paper. Fungal Biol Biotechnol. 2020;7:5.
49. Švarc A, Blažević ZF, Vasić-Rački Đ, Charnock SJ, Presečki AV. A multi-enzyme strategy for the production of a highly valuable lactonized Statin side-chain precursor. Chem Eng Res Des. 2020;164:35–45.
50. Sun T, Wang R, Sun D, Li S, Xu H, Qiu Y, Lei P, Sun L, Xu X, Zhu Y. High-efficiency production of *Tremella aurantialba* polysaccharide through basidiospore fermentation. Bioresour Technol. 2020;318:124268.
51. Qiu X, Gong L, Xin X, An F. Enhancement of emodin production by medium optimization and KH(2)PO(4) supplementation in submerged fermentation of marine-derived Aspergillus favipes HN4-13. Mar Drugs. 2021;19(8):421.
52. Singh S, Arya SK, Krishania M. Bioprocess optimization for enhanced xylitol synthesis by new isolate *Meyerozyma caribbica* CP02 using rice straw. Biotechnol Biofuels Bioprod. 2024;17(1):31.
53. Masi A, Stark G, Pfnier J, Mach RL, Mach-Aigner AR. Exploration of *Trichoderma Reesei* as an alternative host for erythritol production. Biotechnol Biofuels Bioprod. 2024;17(1):90.
54. Nguyen HT, Neelakadan AK, Quach TN, Valliyodan B, Kumar R, Zhang Z, Nguyen HT. Molecular characterization of Glycine max squalene synthase genes in seed phytosterol biosynthesis. Plant Physiol Biochem. 2013;73:23–32.
55. Liang S, Jiang W, Song Y, Zhou SF. Improvement and Metabolomics-Based analysis of d-Lactic acid production from Agro-Industrial wastes by *Lactobacillus delbrueckii* submitted to adaptive laboratory evolution. J Agric Food Chem. 2020;68(29):7660–9.
56. Zhang Y, Vadlani PV, Kumar A, Hardwidge PR, Govind R, Tanaka T, Kondo A. Enhanced D-lactic acid production from renewable resources using engineered *Lactobacillus plantarum*. Appl Microbiol Biotechnol. 2016;100(1):279–88.
57. Cheng Z, He BB, Lei K, Gao Y, Shi Y, Zhong Z, Liu H, Liu R, Zhang H, Wu S, et al. Rule-based omics mining reveals antimicrobial macrocyclic peptides against drug-resistant clinical isolates. Nat Commun. 2024;15(1):4901.
58. Liu Q, Zhang D, Xu Y, Gao S, Gong Y, Cai X, Yao M, Yang X. Cloning and functional characterization of the polyketide synthases based on genome mining of *Preussia isomera* XL-1326. Front Microbiol. 2022;13:819086.
59. Yang YL, Zhang S, Ma K, Xu Y, Tao Q, Chen Y, Chen J, Guo S, Ren J, Wang W, et al. Discovery and characterization of a new family of diterpene cyclases in Bacteria and Fungi. Angew Chem Int Ed Engl. 2017;56(17):4749–52.
60. Chen R, Feng T, Li M, Zhang X, He J, Hu B, Deng Z, Liu T, Liu JK, Wang X, Bian G. Characterization of Tremulane sesquiterpene synthase from the basidiomycete *Irpex lacteus*. Org Lett. 2022;24(31):5669–73.
61. Karlsson M, Durling MB, Choi J, Kosawang C, Lackner G, Tzelepis GD, Nygren K, Dubey MK, Kamou N, Levasseur A, et al. Insights on the evolution of mycoparasitism from the genome of *Clonostachys rosea*. Genome Biol Evol. 2015;7(2):465–80.
62. Sun ZB, Sun MH, Li SD. Draft genome sequence of mycoparasite *Clonostachys rosea* strain 67– 1. Genome Announc. 2015;3(3):e00546–15.
63. Dinday S, Ghosh S. Recent advances in triterpenoid pathway Elucidation and engineering. Biotechnol Adv. 2023;68:108214.
64. Tao H, Lauterbach L, Bian G, Chen R, Hou A, Mori T, Cheng S, Hu B, Lu L, Mu X, et al. Discovery of non-squalene triterpenes. Nature. 2022;606(7913):414–9.
65. Greule A, Stok JE, De Voss JJ, Cryle MJ. Unrivalled diversity: the many roles and reactions of bacterial cytochromes P450 in secondary metabolism. Nat Prod Rep. 2018;35(8):757–91.
66. Cao Z, Li S, Lv J, Gao H, Chen G, Awakawa T, Abe I, Yao X, Hu D. Biosynthesis of clinically used antibiotic fusidic acid and identification of two short-chain dehydrogenase/reductases with Converse stereoselectivity. Acta Pharm Sin B. 2019;9(2):433–42.
67. Cao ZQ, Lv JM, Liu Q, Qin SY, Chen GD, Dai P, Zhong Y, Gao H, Yao XS, Hu D. Biosynthetic study of cephalosporin P(1) reveals a multifunctional P450 enzyme and a Site-Selective acetyltransferase. ACS Chem Biol. 2020;15(1):44–51.
68. Lv JM, Hu D, Gao H, Kushiro T, Awakawa T, Chen GD, Wang CX, Abe I, Yao XS. Biosynthesis of helvolic acid and identification of an unusual C-4-demethylation process distinct from sterol biosynthesis. Nat Commun. 2017;8(1):1644.
69. Ma K, Zhang P, Tao Q, Keller NP, Yang Y, Yin WB, Liu H. Characterization and biosynthesis of a rare fungal Hopane-Type triterpenoid glycoside involved in the antistress property of *Aspergillus fumigatus*. Org Lett. 2019;21(9):3252–6.
70. Yang C, Li W, Li C, Zhou Z, Xiao Y, Yan X. Metabolism of Ganoderic acids by a *Ganoderma lucidum* cytochrome P450 and the 3-keto sterol reductase ERG27 from yeast. Phytochemistry. 2018;155:83–92.
71. Tamura K, Seki H, Suzuki H, Kojoma M, Saito K, Muranaka T. *CYP716A179* functions as a triterpene C-28 oxidase in tissue-cultured stolons of *Glycyrrhiza uralensis*. Plant Cell Rep. 2017;36(3):437–45.
72. Yasumoto S, Fukushima EO, Seki H, Muranaka T. Novel triterpene oxidizing activity of *Arabidopsis thaliana* CYP716A subfamily enzymes. FEBS Lett. 2016;590(4):533–40.

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