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Research Article

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Soybean protein isolate modulates monacolin K accumulation in a high producing *Monascus purpureus* mutant obtained by ARTP mutagenesis

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Abstract: Atmospheric and room-temperature plasma (ARTP) mutagenesis of *Monascus purpureus* 2SH was used to obtain a high-monacolin K (MK)-producing mutant, Y1. The mutant was subsequently used to investigate its response to soybean protein isolate (SPI) supplementation and the potential underlying regulatory mechanisms. Y1 exhibited a stable high-MK-producing phenotype and a favorable safety profile. Different SPI supplementation levels markedly affected MK accumulation, with 3% SPI producing the highest MK content of 19.38 mg/g, 2.35 times that of the unsupplemented control (CK). Transcriptomic analysis of Y1 treated with 3% SPI versus CK identified 1,962 differentially expressed genes (DEGs), including 1,215 upregulated and 747 downregulated genes. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses showed that SPI-responsive DEGs were primarily associated with carbohydrate metabolism, glycolysis/gluconeogenesis, and coenzyme metabolism. The carbohydrate metabolism-related genes *MAP00_006614* and *MAP00_007656* were upregulated, suggesting enhanced carbohydrate utilization and central carbon metabolism following SPI supplementation. In addition, the NADP-dependent malic enzyme gene *MAP00_008064* was upregulated, indicating that reducing power supply and

pyruvate-related metabolism may contribute to MK accumulation. Overall, Y1 showed a pronounced response to SPI supplementation, and SPI may promote MK accumulation by modulating central carbon and coenzyme metabolism. These findings provide insights into the nutritional regulation of MK production and a theoretical basis for improving MK production by *Monascus*.

Keywords: *Monascus purpureus*; monacolin K; soybean protein isolate; atmospheric and room-temperature plasma mutagenesis; transcriptomics

1 Introduction

Monascus spp. are industrially important filamentous fungi that produce a wide range of bioactive metabolites, including monacolins, *Monascus* pigments, and γ-aminobutyric acid (GABA). These metabolites have attracted considerable interest because of their potential applications in functional foods [1–2]. Among them, monacolin K (MK) is a major functional component responsible for the cholesterol-lowering activity of red yeast rice products [3]. Chemically identical to lovastatin, MK competitively inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis. This inhibition suppresses the mevalonate pathway and thereby reduces cholesterol synthesis [4–6]. However, MK productivity varies considerably among *Monascus* strains, and the mycotoxin citrinin may also be produced during fermentation [7]. Therefore, developing strains that combine high MK productivity, genetic stability, and biosafety is essential for improving MK fermentation.

Atmospheric and room-temperature plasma (ARTP) mutagenesis is an efficient physical mutagenesis technique that induces random genetic variation through plasma exposure, thereby expanding the genetic diversity available for microbial strain improvement [8]. This technique has shown considerable potential for the breeding of industrial microorganisms, including *Monascus* strains [9–11]. Li et al. [12] obtained an ARTP-derived mutant that exhibited a 2.3-fold increase in MK production over the parental strain while maintaining good genetic stability. Gao et al. [13] combined ARTP mutagenesis with morphological screening, shake-flask fermentation, and genetic stability assessment to obtain mutants with enhanced pigment and lovastatin

production, with lovastatin yields increasing by 5.14–6.89-fold relative to the original strain.

Nevertheless, MK biosynthesis is influenced not only by the genetic potential of the producing strain but also by medium composition and nutritional conditions [14–16]. Soy protein isolate (SPI) is a protein-rich nutrient source that can provide peptides and amino acids during fermentation, thereby supporting microbial growth and secondary metabolite biosynthesis [17]. Zhang et al. [18] reported that appropriate SPI supplementation promoted mycelial growth and increased MK production in *Monascus pilosus* MS-1. Furthermore, integrated transcriptomic and metabolomic analyses conducted by Qin et al. [19] showed that SPI treatment affected pathways related to nitrogen utilization, amino acid metabolism, glycolysis, pyruvate metabolism, and the tricarboxylic acid (TCA) cycle. SPI treatment also altered the expression of several genes associated with MK biosynthesis.

However, *Monascus* strains differ substantially in their genetic backgrounds and metabolic characteristics. High-MK-producing mutants generated through mutagenesis may possess altered metabolic pathways and regulatory networks and may therefore respond differently to nutrient supplementation. Consequently, the molecular mechanisms underlying SPI-mediated enhancement of MK production in mutagenesis-derived high-producing strains remain poorly understood.

To address this knowledge gap, *Monascus purpureus* 2SH was used as the parental strain in this study, and a high-MK-producing mutant was obtained through ARTP mutagenesis. The effect of SPI supplementation on MK accumulation in the mutant strain was subsequently evaluated. Comparative transcriptomic analysis was further performed to explore the potential molecular mechanisms underlying the SPI-mediated enhancement of MK production. These findings provide a basis for developing high-yielding, stable, and safe *Monascus* strains and for optimizing nutrient-based strategies for MK fermentation.

2 Materials and Methods

2.1 Experimental materials

(1) Parental strain

The parental strain *Monascus purpureus* 2SH was maintained in our laboratory.

(2) Culture media

PDA medium: Potato (200 g/L), glucose (20 g/L), and agar (20 g/L) were added to distilled water to a final volume of 1 L. The medium was sterilized at 121 °C for 30 min.

Liquid seed medium: Anhydrous glucose (60 g/L), tryptone (25 g/L), corn starch (10 g/L), K_2HPO_4 (1 g/L), and $MgSO_4 \cdot 7H_2O$ (1 g/L) were added to distilled water to a final volume of 1 L. The medium was sterilized at 121 °C for 20 min.

Basal fermentation medium: Peptone (2%), glycerol (2.5%), and acetic acid (0.2%) were used as the basal fermentation medium. The medium was sterilized at 121 °C for 20 min.

Soybean protein isolate (SPI) was purchased from Linyi Shansong Biological Products Co., Ltd. (Linyi, China), with a protein content of ≥90%. Monacolin K and citrinin reference standards were purchased from Sigma-Aldrich (USA).

2.2 Instruments and equipment

The main instruments and equipment used in this study are listed in Table 1.

Table 1 Main instruments and equipment used in this study

Instrument	Manufacturer
Electric blast drying oven	Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory, China
Biochemical incubator	Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory, China
High-performance liquid chromatography (HPLC) system	Shimadzu Corporation, Japan
High-speed centrifuge	Thermo Fisher Scientific, USA
Laminar flow cabinet	Heal Force Bio-Meditech Holdings Limited, China
Analytical balance	Sartorius Scientific Instruments (Beijing) Co., Ltd., China

Constant temperature shaking incubator	Zhichu Instrument Co., Ltd., Shanghai, China
Autoclave	Zhimei Instrument Co., Ltd., Xiamen, China

2.3 ARTP mutagenesis and mutant strain screening

ARTP mutagenesis treatment. A fresh spore suspension of *Monascus purpureus* was prepared at a concentration of 1 × 10^8 spores/mL. An aliquot of the suspension was evenly spread onto sterile glass slides. The slides were then subjected to atmospheric and room-temperature plasma (ARTP) mutagenesis at a radio-frequency power of 120 W and an airflow rate of 10 standard liters per minute (SLM). The exposure times were set to 0, 20, 40, 60, 80, 100, 120, 140, and 160 s, with 0 s serving as the untreated control.

After ARTP treatment, the spores were recovered from the glass slides using sterile water. The resulting suspensions were serially diluted and spread onto potato dextrose agar (PDA) plates for cultivation. After incubation, the colonies in each treatment group were counted. The spore lethality rate at each exposure time was calculated relative to the untreated control, and a lethality curve was plotted. Based on the lethality profile, an appropriate ARTP exposure time was selected for the subsequent screening of high-MK-producing mutants.

Mutant strain screening. The ARTP-treated spore suspensions were serially diluted and spread onto PDA plates. The parental strain 2SH was cultured under the same conditions and used as the control. Preliminary phenotypic screening was performed based on colony growth, colony size, pigmentation, and morphological characteristics. The MK content of the solid-state fermentation products was used as the primary screening criterion for selecting high-MK-producing mutants. The stability of the MK-producing phenotype was subsequently evaluated by serial subculturing followed by solid-state fermentation assays across successive generations.

2.4 Preparation of seed culture

The selected mutant strain Y1 was inoculated onto PDA slants and activated at 32 °C for 3 days. The mycelial biomass on the PDA slants was washed with sterile water and vigorously shaken to obtain a homogeneous suspension. The suspension was then filtered through six layers of sterile gauze to remove mycelial fragments and medium residues, yielding a spore suspension. The spore concentration was adjusted to approximately 1 × 10^8 spores/mL.

The spore suspension was inoculated into the seed medium at an inoculation ratio of 10% (v/v) and incubated at 35 °C with shaking at 160 rpm for 48 h. The resulting culture was used as the seed culture of *Monascus purpureus* Y1.

2.5 Optimization of SPI supplementation

Rice was used as the substrate for solid-state fermentation. The rice was washed and soaked in water for 12 h, after which excess surface water was drained. The soaked rice was steamed for 15 min to achieve sufficient gelatinization while preserving the integrity and loose structure of the grains. Before sterilization, the initial moisture content of the complete fermentation substrate, comprising the rice, soybean protein isolate (SPI), and basal fermentation medium, was adjusted to 40%.

SPI was added at concentrations of 2.0%, 2.5%, 3.0%, 3.5%, and 4.0% (w/w), based on the mass of rice. The basal fermentation medium without SPI supplementation served as the control. After thorough mixing, 200 g of each treatment mixture was transferred into a 1,000-mL fermentation bottle and sterilized at 121 °C for 20 min.

After cooling to room temperature, the sterilized substrates were inoculated with the seed culture of the high-MK-producing mutant strain Y1 at an inoculation ratio of 15% (v/w), calculated based on the mass of rice.Fermentation was conducted at 32 °C for the first 3 days and then continued at 23 °C until day 30. At the end of fermentation, the fermented rice samples were dried at 50 °C to a constant weight, milled, and sieved. The MK content was subsequently determined to evaluate the effects of different SPI supplementation levels on MK accumulation. Each treatment was conducted with three independent replicates.

2.6 Determination of Monacolin K Content by HPLC

Monacolin K (MK) content was determined by high-performance liquid chromatography (HPLC) according to the method described in the Chinese Pharmacopoeia (2020 edition) [20], with minor modifications. Solid-state fermentation samples were dried and ground into powder. The powdered samples were extracted with 75% (v/v) ethanol by ultrasonic-assisted extraction for 1 h and subsequently centrifuged at 7,000 rpm for 6 min. The resulting supernatant was collected for HPLC analysis.

Chromatographic separation was performed on a C18 column (250 mm × 4.6 mm, 5 μm) using methanol–water (75:25, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The column temperature was maintained at 30°C, and the detection wavelength was set at 237 nm. The injection volume was 10 μL. A calibration curve was constructed using a series of MK standard solutions, and the MK content of each sample was quantified based on the corresponding peak area.

2.7 Determination of citrinin content

The content of citrinin (CIT) was determined according to the method described in the Chinese national standard *Determination of Citrinin in Foods* (GB 5009.222–2016) [21]. The samples were ground and extracted with an acetonitrile-water solution using ultrasonic-assisted extraction. After centrifugation, the supernatant was filtered through a 0.22 μm membrane filter and analyzed by HPLC. A calibration curve was established using citrinin standard solutions, and the CIT content in samples was calculated accordingly.

2.8 Transcriptome sequencing and bioinformatics analysis

Based on the effects of SPI supplementation on MK accumulation, the 3% SPI treatment was selected for transcriptome analysis, with the treatment without SPI serving as the control. Three biological replicates were prepared for each group.

After solid-state fermentation, the red yeast rice samples were suspended in sterile water and shaken vigorously to separate the fungal biomass from the substrate. The biomass was collected by centrifugation at 5,000 rpm for 10 min, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Total RNA extraction, RNA quality assessment, library preparation, and

transcriptome sequencing were performed by Jiangsu Sanshu Biotechnology Co., Ltd. (China). Raw reads were quality-filtered using fastp, and the resulting clean reads were aligned to the *Monascus purpureus* reference genome (GCA_025999795.1) using STAR. Gene-level read counts were obtained using featureCounts. Pearson correlation analysis and principal component analysis (PCA) were used to assess relationships among samples. Differentially expressed genes (DEGs) were identified using DESeq2, with a raw P value < 0.05 and $|\log_2(\text{fold change})| > 1$ as the selection criteria. GO and KEGG enrichment analyses were performed using over-representation analysis (ORA) based on a hypergeometric test. FDR-adjusted P values (padj) < 0.05 were considered statistically significant for enriched terms and pathways. In the enrichment bubble plots, bubble color represents $-\log_{10}(\text{padj})$.

2.9 Statistical analysis

Unless otherwise stated, experiments were performed with three independent replicates, and results are presented as mean ± standard deviation (SD). Data were processed using Microsoft Excel, and statistical analyses and figure preparation were performed using Origin 2026 (OriginLab Corporation, Northampton, MA, USA). Differences among groups were assessed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test. A P value < 0.05 was considered statistically significant. In tables, means sharing the same superscript letter within a column were not significantly different ($P \geq 0.05$).

3 Results

3.1 ARTP mutagenesis analysis

The lethality of the parental strain 2SH at different ARTP treatment durations is shown in Fig. 1. Lethality increased as the treatment duration was extended. It rose rapidly from 0% at 0 s to approximately 76% at 60 s, then reached approximately 86% and 91% at 80 and 100 s, respectively. The rate of increase subsequently slowed, and lethality approached 100% at 160 s.

These results show that longer ARTP exposure reduced the survival of strain 2SH. Previous studies have suggested approximately 90% lethality as a reference for selecting ARTP mutagenesis conditions [22]. In this study, treatment for 100 s

resulted in approximately 91% lethality while retaining viable cells for subsequent mutant screening. Therefore, 100 s was selected as the ARTP treatment duration for the following experiments.

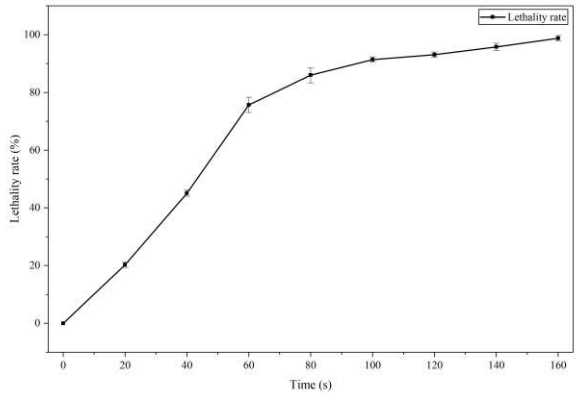


Fig. 1 Effect of ARTP treatment duration on the lethality of the parental strain 2SH

3.2 Screening of high-MK-producing mutant strains

Following ARTP mutagenesis, 12 candidate mutant strains were obtained and evaluated for MK production under solid-state fermentation conditions. As shown in Fig. 2, the strains differed significantly in MK content. Seven mutants produced more MK than the parental strain 2SH, accounting for 58.3% of the candidate strains, whereas the remaining mutants produced less MK than 2SH. This distribution suggests that ARTP mutagenesis altered MK production in different directions, which is consistent with the random nature of mutagenesis and highlights the importance of subsequent screening.

Among the candidate strains, Y1 showed the highest MK content, reaching 7.28 mg/g. This value was 1.68 times that of the parental strain 2SH, corresponding to a 68.1% increase. Y1 therefore exhibited the most prominent high-MK-producing phenotype and was selected for subsequent evaluation of production stability during serial subculture and fermentation performance.

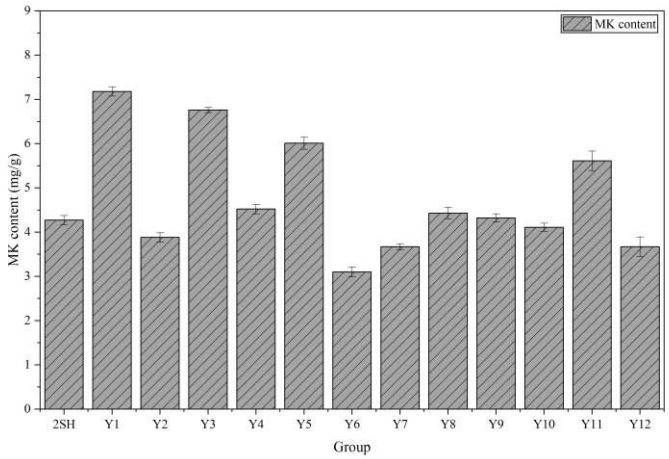


Fig. 2 MK production by ARTP-derived mutant strains

3.3 Safety evaluation of mutant strain Y1

Citrinin (CIT) is a nephrotoxic secondary metabolite that may be produced by certain Monascus strains during fermentation. Its presence is therefore an important indicator for evaluating the safety of red yeast rice products. After screening the high-MK-producing mutants, CIT accumulation in strain Y1 was further assessed.

Table 2 Citrinin content in the parental strain 2SH and mutant strain Y1

Strain name	Citrinin content (μg kg^{-1})
2SH	n.d.
Y1	n.d.

Note: n.d. indicates not detected.

As shown in Table 2, CIT was not detected (n.d.) in either the parental strain 2SH or mutant strain Y1 under the conditions used in this study. Combined with the MK results, Y1 showed an MK content 1.68 times that of 2SH, whereas its CIT content remained below the detection limit. These results suggest that Y1 exhibited enhanced MK accumulation without detectable CIT under the tested fermentation conditions.

Overall, Y1 showed a favorable preliminary production profile, characterized by increased MK accumulation and no detectable CIT. Therefore, Y1 was selected for subsequent solid-state fermentation optimization and investigation of the effects of SPI supplementation.

3.4 Stability analysis of MK-producing phenotype in strain Y1

Strain Y1 was serially subcultured for five generations. Each generation was subjected to solid-state fermentation under identical cultivation conditions to evaluate the stability of its MK-producing phenotype. The results are presented in Table 3.

Table 3 Genetic stability of MK production in the *Monascus purpureus* mutant Y1

Strain	Subculture generation	Lactone-form MK (mg/g)	Acid-form MK (mg/g)	Total MK (mg/g)
Y1	1	2.15 ± 0.27[a]	5.14 ± 0.45[a]	7.29 ± 0.72[a]
	2	2.12 ± 0.32[a]	5.16 ± 0.46[a]	7.28 ± 0.78[a]
	3	2.14 ± 0.31[a]	5.12 ± 0.51[a]	7.26 ± 0.82[a]
	4	2.13 ± 0.33[a]	5.11 ± 0.42[a]	7.24 ± 0.75[a]
	5	2.11 ± 0.35[a]	5.12 ± 0.43[a]	7.23 ± 0.78[a]

Note: Values in the same column sharing the same letter are not significantly different ($P > 0.05$).

Across the five generations, the contents of lactone-form MK, acid-form MK, and total MK remained relatively stable. Total MK was calculated as the sum of lactone-form and acid-form MK. No significant differences were detected among generations ($P > 0.05$). The total MK content decreased slightly from 7.29 ± 0.72 mg/g in the first generation to 7.23 ± 0.78 mg/g in the fifth generation, corresponding to a decrease of only 0.82%. These results indicate that strain Y1 maintained a stable MK-producing phenotype during five consecutive generations of subculturing.

Considering its high MK production and the absence of detectable CIT, strain Y1 was selected for subsequent SPI supplementation experiments and transcriptome analysis.

3.5 Effect of different soybean protein isolate supplementation levels on MK content

The effects of different soybean protein isolate (SPI) supplementation levels on MK accumulation by strain Y1 during solid-state fermentation were investigated, and the results are shown in Fig. 3. As the SPI level increased from 2.0% to 3.0%, the MK content increased and reached a maximum of 19.38 mg/g at 3.0% SPI. This value was 2.35 times that of the control group without SPI supplementation (CK). However, further increases in the SPI level to 3.5% and 4.0% resulted in a decrease in MK content. These results indicate that the effect of SPI on MK accumulation was

concentration-dependent within the tested range, with 3.0% SPI producing the highest MK content under the conditions used in this study.

Appropriate SPI supplementation may provide additional nitrogen and amino acids to the fermentation system, thereby supporting fungal growth and secondary metabolite production. Excessive SPI supplementation, however, may alter the nutritional balance of the fermentation substrate and weaken the positive effect of SPI on MK accumulation. Based on these results, the 3.0% SPI treatment was selected for subsequent transcriptome analysis, while the group without SPI supplementation was used as the control.

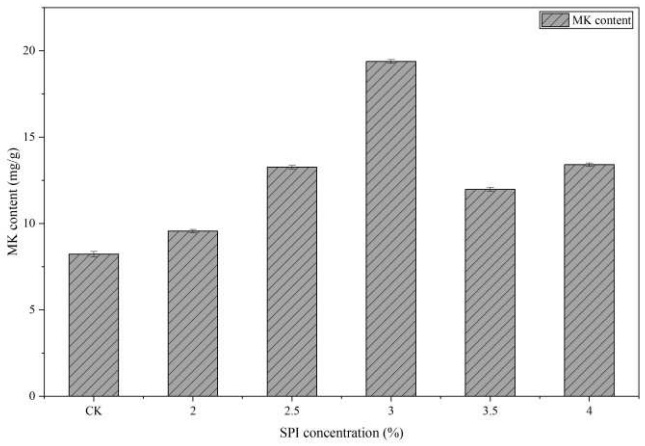


Fig. 3 Effect of SPI supplementation on MK accumulation in red yeast rice fermented by strain Y1

3.6 Transcriptome sequencing and bioinformatics analysis

3.6.1 Evaluation of RNA-seq data quality

The quality of the RNA-seq data generated from the SPI treatment and control groups was evaluated, and the results are presented in Table 4.

Table 4 Quality assessment of RNA-seq data

Sample	Total reads	Total bases(Gb)	Q20	Q30	GC content(%)
D-1	41791068	6.31	98.85%	95.54%	53.05%
D-2	41544946	6.27	98.87%	95.61%	52.48%
D-3	40179276	6.07	98.83%	95.46%	52.55%
D1-1	41367266	6.25	98.95%	95.63%	52.62%
D1-2	36730776	5.55	98.90%	95.52%	53.06%
D1-3	37554524	5.67	98.99%	95.80%	52.67%

Note: D denotes the SPI treatment group, and D1 denotes the control group (CK). The

SPI group was supplemented with 3% SPI, whereas the CK group received no SPI supplementation. Each group included three biological replicates.

As shown in Table 4, each of the six samples yielded 36.73–41.79 million raw reads and 5.55–6.31 Gb of sequencing data. The Q20 values exceeded 98.8% for all samples, while the Q30 values ranged from 95.46% to 95.80%, indicating high base-calling accuracy. The GC content ranged from 52.48% to 53.06%, with only minor variation within and between groups, suggesting a stable base composition across the sequencing data.

Overall, all samples yielded sufficient sequencing data with good quality metrics, meeting the requirements for subsequent reference-genome alignment, gene-expression quantification, and differential-expression analysis.

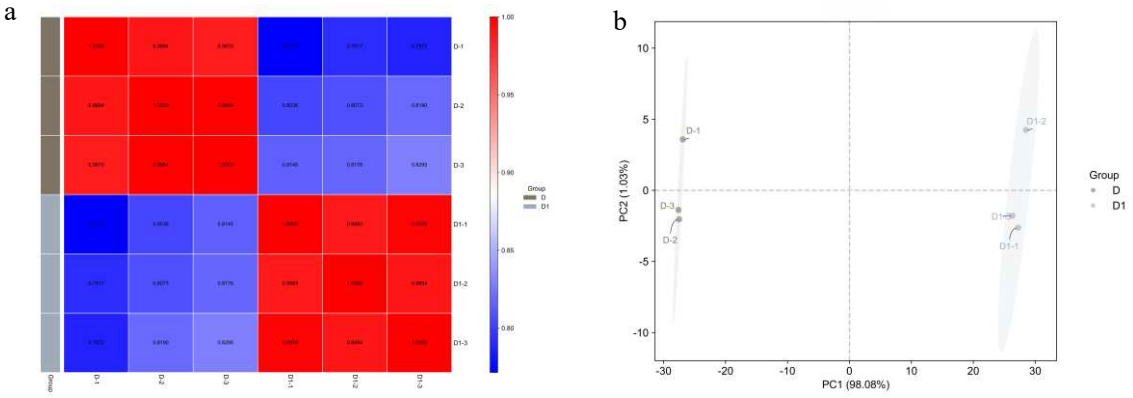


Fig. 4 Pearson correlation analysis and principal component analysis of RNA-seq samples. (a) Pearson correlation analysis among samples. (b) Principal component analysis of the samples

To assess the consistency of the biological replicates and the effects of SPI supplementation on global gene-expression patterns in *Monascus purpureus*, Pearson correlation analysis and principal component analysis (PCA) were performed using the six RNA-seq samples. As shown in Fig. 4a, the within-group correlation coefficients ranged from 0.9870 to 0.9984 for the D group and from 0.9883 to 0.9976 for the D1 group. These values were higher than the between-group correlations, which ranged from 0.7715 to 0.8295. The high within-group correlations indicated good reproducibility among the biological replicates, whereas the lower between-group correlations suggested distinct gene-expression profiles between the two

groups.

PCA showed that PC1 and PC2 explained 98.08% and 1.03% of the total variance, respectively. The samples from the D and D1 groups were clearly separated along PC1, while biological replicates from the same group clustered closely together (Fig. 4b). This separation indicated that SPI supplementation was associated with marked changes in the global transcriptional profile of *M. purpureus*.

Overall, the RNA-seq samples showed high data quality and strong within-group consistency. The distinct separation between the D and D1 groups further indicated that the data were suitable for subsequent differential-expression and functional-enrichment analyses.

3.6.2 Identification and expression pattern analysis of differentially expressed genes in response to SPI treatment

The D1 group without SPI supplementation was used as the control, and differential expression analysis was performed using DESeq2. Genes with an adjusted *p*-value (*padj*) < 0.05 and $|\log_2 FC| \geq 1$ were defined as differentially expressed genes (DEGs). In the D vs. D1 comparison, the D group represented the 3% SPI treatment, and genes with $\log_2 FC > 0$ were considered upregulated in the SPI-treated group. The results are shown in Fig. 5.

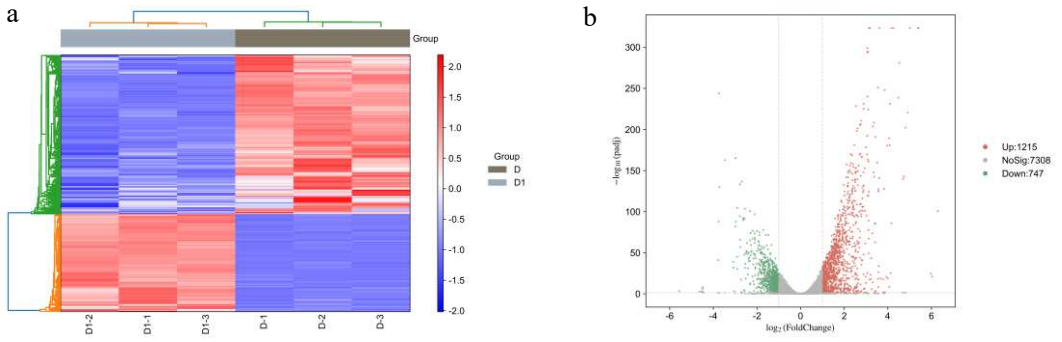


Fig. 5 Differentially expressed genes in Monascus purpureus in response to SPI supplementation. (a) Hierarchical clustering heatmap. (b) Volcano plot

Using the D1 group as the control, genes with $\log_2 FC > 0$ in the D vs. D1 comparison were considered upregulated in the SPI-treated D group. A total of 1,962 differentially expressed genes (DEGs) were identified, including 1,215 upregulated genes and 747 downregulated genes, which accounted for 61.9% and 38.1% of the

total DEGs, respectively (Fig. 5b).

The hierarchical clustering heatmap showed that the samples from the D and D1 groups formed separate clusters, while the three biological replicates within each group displayed consistent expression patterns. Several major gene clusters showed opposite expression trends between the two groups. Some genes were highly expressed in the D group but expressed at lower levels in the D1 group, whereas other genes showed the opposite pattern (Fig. 5a).

The volcano plot showed that the number of upregulated genes was higher than that of downregulated genes, indicating that SPI supplementation induced a broad transcriptional response in *Monascus purpureus*, with upregulation predominating. These findings indicate that 3% SPI supplementation significantly altered the gene-expression profile of *M. purpureus*. The DEGs were subsequently subjected to functional enrichment analysis to identify the metabolic processes potentially involved in SPI-mediated MK accumulation.

3.6.3 GO and KEGG enrichment analyses of differentially expressed genes

To characterize the biological functions and metabolic pathways associated with the DEGs induced by SPI supplementation, GO and KEGG enrichment analyses were performed. Terms related to carbon-source utilization, redox metabolism, precursor supply, and amino acid metabolism were selected from the significantly enriched results for presentation. The results are shown in Fig. 6.

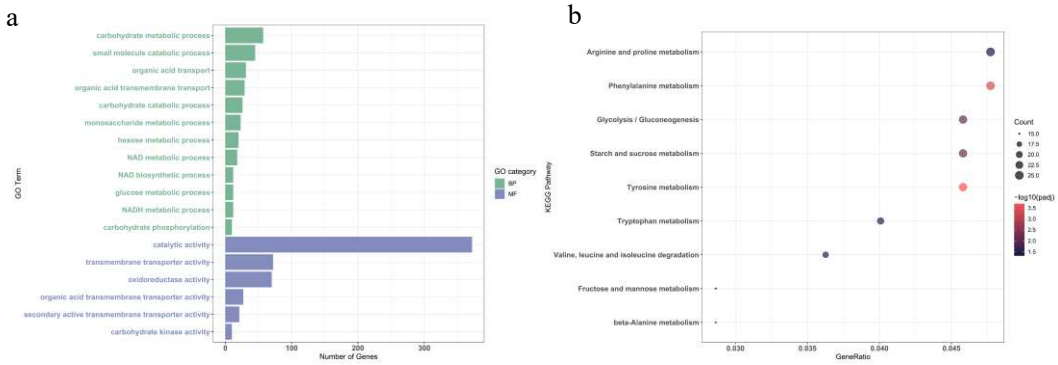


Fig. 6 GO and KEGG enrichment analyses of differentially expressed genes in Monascus purpureus under SPI supplementation. (a) GO enrichment analysis. (b) KEGG pathway enrichment analysis

GO enrichment analysis identified 18 significantly enriched terms, including 12

biological process (BP) terms and 6 molecular function (MF) terms (Fig. 6a). The BP terms were mainly associated with carbohydrate metabolism and catabolism, monosaccharide and hexose metabolism, glucose metabolism, carbohydrate phosphorylation, small-molecule catabolism, NAD/NADH metabolism, organic acid transport, and transmembrane transport. The MF terms mainly included catalytic activity, oxidoreductase activity, carbohydrate kinase activity, transmembrane transporter activity, and organic acid transmembrane transporter activity. These results suggest that the DEGs induced by SPI supplementation were mainly involved in carbon-source utilization, redox cofactor metabolism, and transmembrane transport. No significantly enriched terms were identified in the cellular component (CC) category; therefore, the CC results are not shown.

KEGG enrichment analysis showed that the DEGs were significantly enriched in nine pathways, including glycolysis/gluconeogenesis; starch and sucrose metabolism; fructose and mannose metabolism; phenylalanine metabolism; tyrosine metabolism; tryptophan metabolism; arginine and proline metabolism; β-alanine metabolism; and branched-chain amino acid metabolism (Fig. 6b). Glycolysis/gluconeogenesis, starch and sucrose metabolism, and fructose and mannose metabolism are closely related to carbohydrate degradation and central carbon metabolism. Changes in these pathways may affect the supply of pyruvate, acetyl-CoA, and other precursors required for polyketide biosynthesis. The enrichment of multiple amino acid metabolism pathways also suggests that SPI supplementation altered nitrogen utilization and amino acid metabolic networks in Monascus purpureus.

Taken together, the GO and KEGG results indicate that SPI supplementation mainly affected carbohydrate metabolism, cofactor and redox metabolism, material transport, and amino acid metabolism in M. purpureus. Coordinated changes in these processes may be involved in the regulation of MK accumulation. Because the enrichment analysis was based on all identified DEGs, pathway enrichment indicates only a relative overrepresentation of DEGs in the corresponding pathways. It does not demonstrate that the pathways were globally activated or inhibited. The direction of

regulation should therefore be further evaluated by examining the expression changes of key genes.

3.6.4 Expression pattern analysis of MK accumulation-related genes under SPI treatment

Based on the differential expression results and functional annotations, 20 DEGs associated with carbon-source utilization, precursor and reducing-power supply, polyketide biosynthesis, and secondary-metabolism regulation were selected for expression-pattern analysis. The results are shown in Fig. 7.

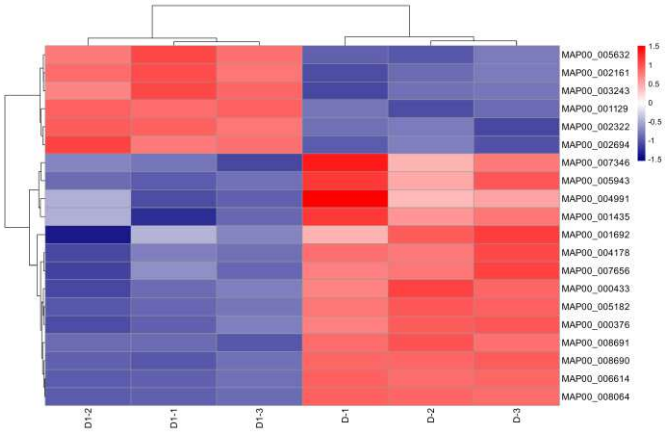


Fig. 7 Hierarchical clustering heatmap of genes associated with MK accumulation under SPI supplementation

The clustering analysis clearly separated the samples from the D (SPI-treated) and D1 (control) groups. The three biological replicates within each group clustered closely together, indicating distinct expression patterns between the two groups and good consistency among replicates. Among the 20 candidate genes, 14 showed relatively higher expression in the D group, whereas 6 showed relatively lower expression.

The genes with higher expression in the D group were mainly associated with carbon-source utilization, glycolysis, redox metabolism, and secondary metabolism. Several genes involved in carbohydrate metabolism, including MAP00_004178, which is related to glucose transport; *MAP00_006614*, encoding glucokinase; *MAP00_007656*, encoding phosphofructokinase; *MAP00_000376*, encoding phosphoglycerate kinase; and *MAP00_000433*, encoding triose phosphate isomerase,

showed increased expression. These changes suggest that SPI supplementation may promote glucose uptake and glycolysis. In addition, *MAP00_008064*, encoding an NADP-dependent malic enzyme; *MAP00_007346*, encoding an NADPH–cytochrome P450 reductase; and *MAP00_008690*, a fatty acid synthase-related gene, were upregulated. This result suggests that SPI supplementation may affect cellular reducing-power supply and lipid metabolism.

By contrast, *MAP00_005632*, *MAP00_002161*, *MAP00_003243*, *MAP00_002322*, *MAP00_002694*, and *MAP00_001129* showed relatively lower expression in the D group. These results indicate that the effects of SPI supplementation on the MK-related metabolic network were not limited to uniform upregulation. Instead, SPI supplementation was also associated with the downregulation of some candidate genes involved in polyketide biosynthesis and secondary-metabolism regulation.

Overall, SPI supplementation may contribute to MK accumulation by reshaping central carbon metabolism, reducing-power supply, precursor allocation, and secondary-metabolism regulation in Monascus purpureus.

4 Discussion

Atmospheric and room-temperature plasma (ARTP) mutagenesis can generate genetic variation in microorganisms, providing an effective approach for obtaining strains with high production of target metabolites. However, because mutagenesis is random, product-specific screening and genetic stability evaluation remain essential for identifying promising strains with practical application potential. In this study, the high-monacolin K (MK)-producing mutant Y1 was obtained from *Monascus purpureus* 2SH through ARTP mutagenesis and screening. The MK content of Y1 reached 7.28 mg/g, which was 1.68 times that of the parental strain. Its MK-producing capacity showed no significant change after five successive passages, and citrinin was not detected under the analytical conditions used in this study, indicating a stable MK-producing phenotype and a favorable safety profile under the conditions examined. Thus, Y1 serves as both a high-MK-producing mutant for further investigation and an

experimental platform for characterizing the effects of nutritional factors on MK accumulation in a high-producing mutant background.

After obtaining a stable high-producing mutant, we further investigated the effects of soybean protein isolate (SPI) supplementation on MK accumulation during solid-state fermentation by Y1. Different SPI supplementation levels markedly affected MK accumulation. At 3% SPI supplementation, the MK content reached 19.38 mg/g, which was 2.35 times that of the unsupplemented control (CK). Further increases in SPI supplementation were associated with a declining trend in MK content, suggesting a concentration-dependent effect of SPI on MK accumulation in Y1. Previous studies have shown that SPI can influence secondary metabolite accumulation in *Monascus*. Qin et al. [19] reported that SPI supplementation increased MK production in a liquid fermentation system using *Monascus pilosus* MS-1, accompanied by changes in the expression of multiple metabolism-related genes. Because the two studies differed in strain background, cultivation system, and fermentation mode, their SPI supplementation levels and magnitudes of change in MK production should not be compared directly. Nevertheless, both studies observed that SPI supplementation modulated MK accumulation in *Monascus*. These findings suggest that SPI may influence MK accumulation by modifying nutrient utilization and the underlying metabolic state, although the specific regulatory responses may depend on the strain background and cultivation system.

To further investigate the potential regulatory mechanisms underlying the effects of SPI on MK accumulation in Y1, transcriptomic analysis was performed on the 3% SPI treatment group. A total of 1,962 differentially expressed genes (DEGs) were identified following SPI treatment, including 1,215 upregulated and 747 downregulated genes. GO and KEGG enrichment analyses indicated that the DEGs were primarily associated with carbohydrate metabolism, glycolysis/gluconeogenesis, coenzyme metabolism, amino acid metabolism, and transmembrane transport, suggesting that SPI supplementation may alter the overall metabolic state of Y1. In particular, *MAP00_006614*, encoding hexokinase, and *MAP00_007656*, encoding phosphofructokinase, were upregulated. Hexokinase catalyzes glucose

phosphorylation, whereas phosphofructokinase catalyzes a key reaction in glycolysis. Their expression changes suggest that SPI treatment may improve carbohydrate utilization efficiency and promote central carbon metabolism, potentially providing metabolic support for MK accumulation.

In addition to genes associated with carbon metabolism, the NADP-dependent malic enzyme gene *MAP00_008064* was significantly upregulated following SPI treatment. This enzyme participates in the conversion of malate to pyruvate and may influence NADPH production. The expression change of *MAP00_008064* therefore suggests that the malic enzyme pathway may contribute to regulating cellular reducing power supply during the response of Y1 to SPI supplementation, potentially supporting secondary metabolic processes associated with MK biosynthesis. Together with the enrichment of coenzyme metabolism and redox-related functional categories in GO analysis, and of glycolysis/gluconeogenesis, starch and sucrose metabolism, and fructose and mannose metabolism in KEGG analysis, these findings suggest that SPI may influence MK accumulation in Y1 primarily through changes in central carbon metabolism and coenzyme metabolic networks.

Consistent with the findings of Qin et al. in *M. pilosus*, this study also observed SPI-induced changes in processes related to glycolysis and central carbon metabolism, suggesting that SPI-promoted MK accumulation may be closely associated with changes in primary metabolic networks. However, Qin et al. reported that enhanced MK accumulation following SPI supplementation was mainly accompanied by increased expression of several MK biosynthesis-related genes, including *mokA*, *mokB*, and *mokC*. The same expression response pattern was not observed in Y1 in the present study. Instead, the NADP-dependent malic enzyme gene *MAP00_008064* was significantly upregulated. This metabolic response has received limited attention in previous studies of SPI-mediated MK accumulation in *Monascus*, suggesting that reducing power supply may be one of the potential metabolic factors involved in the regulation of MK accumulation during the response of Y1 to SPI supplementation. Nevertheless, because this study did not include a transcriptomic comparison of the parental strain 2SH under the same SPI conditions, these response

characteristics cannot be directly attributed to ARTP mutagenesis. Future studies will compare the transcriptional responses of 2SH and Y1 to SPI supplementation to determine whether ARTP mutagenesis reshapes the metabolic responses of *Monascus* to nutritional supplementation.

In addition to SPI, the regulation of MK accumulation by other exogenous nutrients is also closely associated with changes in primary metabolic networks. Zhang et al. [23] and Dong et al. [24] reported that enhanced MK accumulation following exogenous nutrient supplementation was accompanied by changes in pathways related to carbon and energy metabolism. Some studies have further shown that changes in MK production depend not only on the expression of biosynthesis-related genes but also on precursor supply, reducing power availability, and the overall cellular metabolic state. Li et al. [25] and Fang et al. [26] further indicated that secondary metabolite formation often results from coordinated regulation across multiple metabolic modules. Accordingly, the present findings suggest that the effects of SPI on MK accumulation in Y1 may involve coordinated regulation of central carbon metabolism, coenzyme metabolism, reducing power supply, and transport processes, rather than regulation through a single metabolic node.

Taken together, this study demonstrates that ARTP mutagenesis and screening yielded the *M. purpureus* mutant Y1 with high MK-producing capacity and a stable production phenotype, and further characterizes its metabolic responses to SPI supplementation. SPI treatment may provide metabolic support for MK accumulation by modulating central carbon metabolism, coenzyme metabolism, and processes related to reducing power supply. Compared with previous liquid fermentation studies, this work extends SPI-based nutritional regulation to an ARTP-derived high-producing mutant in a rice-based solid-state fermentation system. It also provides preliminary insights into the potential molecular mechanisms underlying the effects of SPI on MK accumulation by characterizing the nutritional responses of this high-producing mutant. Although the present results do not establish that ARTP mutagenesis caused a distinct SPI response pattern, they provide a new perspective on nutritional regulation in high-producing *Monascus* mutants and offer a theoretical

basis for fermentation optimization and the practical application of high-MK-producing strains.

5 Conclusions

In this study, *Monascus purpureus* 2SH was used as the parental strain to obtain the high-MK-producing mutant Y1 through atmospheric and room-temperature plasma (ARTP) mutagenesis. The MK content of Y1 reached 7.28 mg/g, which was 1.68 times that of the parental strain. Y1 maintained stable MK production after five successive generations, and citrinin was not detected under the conditions tested, indicating that Y1 exhibited good stability and a favorable safety profile for MK production. Furthermore, SPI supplementation significantly affected MK accumulation during solid-state fermentation by Y1. Under the conditions tested, 3% SPI resulted in the highest MK content (19.38 mg/g), which was 2.35 times that of the CK group, whereas further increasing the SPI supplementation level resulted in a decreasing trend in MK content. Transcriptomic analysis identified 1,962 differentially expressed genes between the 3% SPI treatment and CK groups, which were mainly associated with carbohydrate metabolism, glycolysis/gluconeogenesis, coenzyme metabolism, and amino acid metabolism. Among these genes, *MAP00_006614*, *MAP00_007656*, and *MAP00_008064* were significantly upregulated. Overall, the results suggest that SPI may promote MK accumulation in Y1 by modulating central carbon metabolism, coenzyme metabolism, and reducing-power supply. This study provides new insights into the metabolic response of high-producing mutants to nutritional supplementation and offers a theoretical basis for improving MK production through strain improvement and nutritional regulation.

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Data availability

The RNA sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number

PRJNA1551636. The associated SRA run accessions are SRR41413508–SRR41413513. The data will be made publicly available upon publication.

Author contributions

Ying Yang: Writing – original draft, Resources, Investigation. **Xingrong Zhang:** Project administration, Methodology. **Yanhong Huang:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Competing interests

The authors declare that they have no competing interests.

Declarations

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

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