Effect of Temperature-Shift and Temperature-Constant Cultivation on the Monacolin K Biosynthetic Gene Cluster Expression in *Monascus* sp.

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Summary

In this study, the effects of temperature-shift (from 30 to 25 °C) and temperature-constant (at 30 °C) cultivation on the mass of *Monascus fuliginosus* CG-6 mycelia and concentration of the produced monacolin K (MK) were monitored. The expression levels of the MK biosynthetic genes of *M. fuliginosus* CG-6 at constant and variable culture temperatures were analysed by real-time quantitative polymerase chain reaction (RT-qPCR). The total protein was collected and determined by liquid chromatography-electrospray ionisation with tandem mass spectrometry (LC-ESI-MS/MS). Results showed that the maximum mycelial mass in temperature-shift cultivation was only 0.477 g of dry cell mass per dish, which was lower than that in temperature-constant cultivation (0.581 g of dry cell mass per dish); however, the maximum concentration of MK in temperature-shift cultivation (34.5 µg/mL) was 16 times higher than that in temperature-constant cultivation at 30 °C (2.11 µg/mL). Gene expression analysis showed that the expression of the MK biosynthetic gene cluster at culture temperature of 25 °C was higher than that at 30 °C, which was similar to the trend of the MK concentration, except for individual *MK B* and *MK C* genes. Analysis of differential protein expression revealed that 2016 proteins were detected by LC-ESI-MS/MS. The expression level of efflux pump protein coded by the *MK I* gene exhibited the same upregulated trend as the expression of *MK I* in temperature-shift cultivation. Temperature-shift cultivation enhanced the expression of proteins in the secondary metabolite production pathway, but suppressed the expression of proteins involved in the mycelial growth.

Key words: Monascus fuliginosus CG-6, monacolin K, temperature, protein analysis, gene analysis

Introduction

Monascus spp. are traditional food fungi that can produce many kinds of secondary metabolites, including pigments, γ-aminobutyric acid and monacolins (*1*). *Monascus* spp. contain statins, which can be used for alternative statin treatment. In particular, monacolin K (MK, also named lovastatin, mevinolin, and Mevacor®) is a strong 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor. HMG-CoA reductase is an enzyme that mediates the rate-limiting reaction of cholesterol biosyn-

thesis. MK can inhibit cholesterol biosynthesis and lower blood cholesterol levels in both humans and animals (*2*). Therefore, it is known as an anticholesterol and antihypotensive agent (*3*).

The culture environment is crucial for filamentous fungi to produce secondary metabolites. Different culture temperatures can influence the production of secondary metabolites. Ahn *et al.* (*4*) proved that the pigment yield at 25 °C is 10 times higher than that at 30 °C, because the lower temperature (25 °C) can reduce the fungal growth

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rate. Tsukahara *et al.* (*5*) separated the growth phase and lovastatin production phase by lowering the temperature from 30 to 25 °C. *Monascus pilosus* NBRC 4520 was cultured at 30 °C for 4 days, followed by additional 17 days of incubation at 25 °C. Results showed that *M. pilosus* hardly produced MK during temperature-constant cultivation at 30 °C, but efficient production was observed after lowering the culture temperature from 30 to 25 °C. However, the reason why the shift in temperature led to high MK production remains unclear.

To improve our understanding of the action mechanism underlying MK production in *Monascus* sp. *via* temperature-shift cultivation, a detailed study of the effect of temperature-shift and temperature-constant cultivation on MK production and the possible mechanism is necessary. In this study, the mycelial mass and MK concentration during temperature-shift and temperature-constant cultivation were monitored. The expression levels of the MK biosynthetic genes were measured by real-time quantitative polymerase chain reaction (RT-qPCR). The protein expression of mycelia was analysed by LC-ESI-MS/MS. This study aims to investigate the relationship between temperature and the MK biosynthetic gene cluster. Several secondary metabolites and metabolism growth--related proteins that differ in amounts during temperature-shift and temperature-constant cultivation were identified.

Materials and Methods

Strains and culture conditions

Preserved *Monascus fuliginosus* CG-6, bought from China Center of Industrial Culture Collection (CICC), was activated on malt extract agar for 6 days at 30 °C. Spores were harvested with 2 mL of sterile water and inoculated into 100 mL of seed medium (in %): glucose 6, peptone 2, KH_2PO_4 1, $NaNO_3$ 1 and $MgSO_4 \cdot 7H_2O$ 0.5 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, PR China) in 250-mL flasks. The culture was incubated at 30 °C for 48 h with shaking at 180 rpm. To measure the biomass and MK production by *Monascus fuliginosus* CG-6, 10 % fungal liquid was inoculated on seed medium containing 4 % cellophane agar and the mycelium was cultured at 30 °C for 6 days. The cultures were subsequently divided into two groups: one group was cultured at 30 °C for 15 days, and the other at 25 °C for 15 days. The spore concentration was adjusted to approx. 10^6 spore/mL by haemocytometer.

Determination of biomass

The mycelium biomass at different culture temperatures was determined by removing the cellophane to recover the mycelia of the two groups. The biomass yield in solid seed culture medium was determined gravimetrically after drying at 50 °C overnight to a constant mass. The biomass was determined every 3 days. All samples were determined at least three times.

Extraction and HPLC analysis

The sample was dried and ground into a powder. Approximately 0.5 g of powder was extracted by 3 mL of 75 % ethanol for 30 min in an ultrasonic bath and then centrifuged at 2150×*g* (model Anke TDL-5-A; Shanghai Anting Scientific Instrument Factory Co., Ltd., Shanghai, PR China) for 15 min. Samples were prepared in triplicate. The total supernatant was pooled and passed through 0.22-µm RC filters (ANPEL Laboratory Technologies Inc., Shanghai, PR China).

MK was detected by HPLC-DAD (Agilent Technologies, Chengdu, PR China) at 270 nm. An Eclipse XDB-C18 column (4.6 mm×150 mm, 5 µm; Agilent Technologies) was used at 25 °C, and isocratic elution was performed for 30 min using acetonitrile/water (containing 0.05 % methanoic acid) 60:40, by volume at 1 mL/min. Standards from Sigma-Aldrich (St. Louis, MO, USA) were used. The MK concentration was determined every 3 days. All experiments were performed in triplicate.

Gene expression analysis with RT-qPCR

The mycelia were obtained at two different culture temperatures (25 and 30 °C) when the MK concentration peaked. Total mycelial RNA was extracted by Plant RNA kit (Omega, Shanghai, PR China), and cDNA was synthesised by PrimeScript 1st strand cDNA synthesis kit (Takara Bio Inc., Dalian, PR China) with Oligo dT Primer 15. RT-qPCR was conducted to monitor gene expression levels using the SYBR Premix Ex Taq II (Takara Bio Inc.). Primers for *MK A*, *MK B*, *MK C*, *MK D*, *MK E*, *MK F*, *MK G*, *MK H* and *MK I* genes (GenBank accession no. DQ176595.1) (*6*) and the β-actin gene (GenBank accession no. AJ417880) (*7*) were designed by Primer Premier v. 5 software (*8*) to amplify a portion of the nine genes (Table 1). RT-qPCR was performed using Stratagen Mx3000P

Table 1. The primers used in this study in RT-qPCR designed by Primer Premier v. 5 software (*8*)

Primer	Sequence (5'-3')	Product size/bp
β-actin-F	AGTCCAACAGGGAGAAGATG	132
β-actin-R	CACCAGAGTCAAGCACGATA	
MK A-F	GAACCATCGCCGACAAAT	179
MK A-R	AACAAGGTCCAAGTCATCCAG	
MK B-F	ACAGTGTAGAGGGAGGTGTAAG	159
MK B-R	AATCGGAGACCGTGTATGT	
MK C-F	TGAGCCGAAGAAGTACGACC	152
MK C-R	CCTTGGAGGCGAAGAACC	
MK D-F	ATGCGGGAGGAGGTAGGCT	198
MK D-R	CCGATTCGGGAAATGATGC	
MK E-F	ATCCTCCACGAGCCTTGCC	194
MK E-R	GCCGCTATGTCTCCCTGAAC	
MK F-F	AAACGGAGAAGCAGATGAACC	103
MK F-R	ATCCCACCAAGCCCAAAA	
MK G-F	ACACGCCGAGACGGTAGA	161
MK G-R	CGAGCCCGAATACAAAGG	
MK H-F	GTCTGCCCACTCCTCCTT	190
MK H-R	CTTCAACCCGAATCTCCAC	
MK I-F	CAGAACCCAAACACCACC	109
MK I-R	TGATCTCACTTGCGTTAGAAT	

(Agilent Technologies) with the following cycling program: 95 °C for 30 s, followed by a three-step PCR (40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s) and denaturation curve analysis (95 °C for 15 s, annealing at 58 °C for 30 s, collecting the denaturation curve from 58 to 95 °C, finally at 95 °C for 15 s). Relative expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method (*9*). All values were normalised using the reference expression level of the β-actin gene. The gene expression level of the mycelia cultured at 30 °C was used as a calibrator.

Sample preparation and protein extraction

Monascus fuliginosus CG-6 samples were ground in liquid nitrogen and lysed in 8 M urea supplemented with 1 % nonyl phenoxypolyethoxyethanol (NP-40) (Beyotime, Shanghai, PR China), 2 mM ethylenediaminetetraacetic acid (EDTA) (Sinopharm Chemical Reagent Co., Ltd.), 5 mM dithiothreitol (DTT) (Thermo Fisher Scientific, Shanghai, PR China) and protease inhibitor cocktail. The samples were lysed by sonication with 12 short bursts of 10 s followed by intervals of 30 s for cooling. Unbroken cell and debris were removed by centrifugation (Backman Coulter, Beijing, PR China) at 4 °C and 2960×*g* for 10 min. The protein content in the supernatant was determined with a 2D Quant kit (GE Healthcare, Beijing, PR China). An equal amount of protein was reduced with 5 mM DTT and alkylated with 25 mM iodoacetamide (IAM) (Sinopharm Chemical Reagent Co., Ltd.). The precipitate was washed three times with cold acetone after centrifugation, suspended in 0.1 M tetraethylammonium bromide (TEAB) (Sinopharm Chemical Reagent Co., Ltd.) and digested with trypsin (Promega, Madison, WI, USA) at an enzyme--to-substrate ratio of 1:50 for 12 h at 37 °C. Digestion was terminated with 1 % trifluoroacetic acid (TFA) (Thermo Fisher Scientific) and the resulting peptides were purified with a Strata X C18 SPE column (Phenomenex, Guangzhou, PR China) and dried in Scanvac (Labogene, Shanghai, PR China).

LC-ESI-MS/MS analysis by Q Exactive plus

The lyophilised peptides were resuspended in buffer A (2 % acetonitrile (ACN) and 0.1 % formic acid (FA)) (Thermo Fisher Scientific), loaded onto an Acclaim PepMap 100 C18 trap column (75 μm×0.02 cm; Dionex, Shanghai, PR China) by Ultimate 3000 nano ultra-performance liquid chromatograph (UPLC) (Dionex), and eluted onto an Acclaim PepMap rapid separation liquid chromatograph (RSLC) C18 analytical column (75 μm×0.25 cm; Dionex). A 90-minute linear gradient was run at 0.3 mL/min, starting from 8 to 35 % B (80 % ACN and 0.1 % FA), followed by 4-minute gradient to 80 % B, and maintained at 80 % B for 8 min.

The peptides were subjected to a nanospray ionisation source in Q Exactive plus mass spectrometer (Thermo Fisher Scientific) coupled online to UPLC. Intact peptides were detected in the Orbitrap (Thermo Fisher Scientific) at a resolution of 70 000 and the *m/z* scan range from 350 to 1400 Da. The 20 most intense peptides above a threshold ion counts of 2E4 were sequentially isolated and frag-

mented for MS/MS using 27 % normalised collision energy (NCE) (Thermo Fisher Scientific). The dynamic exclusion was 30 s. Ion fragments were detected in the Orbitrap (Thermo Fisher Scientific) at a resolution of 17 500. The applied electrospray voltage was 2.0 kV. Automatic gain control was used to prevent overfilling of the ion trap; 3E6 and 1E5 ions were accumulated for the generation of MS and MS/MS spectra, respectively.

Database search

MaxQuant software (*10*) with an integrated Andromeda (*11*) search engine v. 1.3.0.5 was used to identify the protein. Tandem mass spectra were searched against a database of predicted *Monascus purpureus* strain YY-1 (*12*) proteins (7491 sequences). Trypsin/P was specified as a cleavage enzyme allowing up to two missing cleavages, three modifications per peptide and five charges. Mass error was set to 6 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation on Cys was specified as fixed modification. Oxidation on Met and acetylation on the protein N-terminal were specified as variable modifications. False discovery rate thresholds for protein, peptide and modification site were specified at 0.01. The minimum peptide length was 6. The protein hit of 'reversed', 'contaminant ' or 'only identified by site' was removed.

Protein quantitation

Label-free quantification of identified proteins was performed by MaxLFQ (*13*) integrated into MaxQuant software. In addition to the parameters set above, LFQ quantification was enabled by checking 'Match between runs ', 'LFQ ' and 'iBAQ '.

After MaxQuant processing, the proteinGroup.txt file was loaded and analysed by Perseus v. 1.5.0.31 software (*14*). Briefly, the LFQ intensity values of two samples were logarithmically transformed. The missing LFQ value was replaced by normal distribution to simulate the background LFQ intensity level. The relative protein ratio was calculated by exponential transformation of the different values between the logarithmically transformed LFQ values of two samples.

Graphics programme

Origin v. 8 software (*15*) was used in this study to draw the figures. Data were subjected to ANOVA using Excel v. 10 (*16*).

Results and Discussion

Mycelial biomass

Biomass was measured at an interval of 3 days (Fig. 1). Cell growth was slow in temperature-shift cultivation. The maximum biomass was only 0.477 g of dry cell mass per dish on the 18th day of dish fermentation. The maximum dry cell mass in temperature-constant cultivation was 0.581 g per dish on the 21st day, which was higher than that in temperature-shift cultivation.
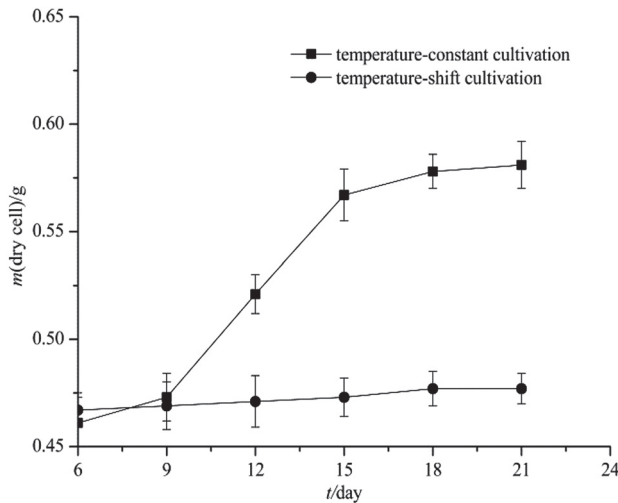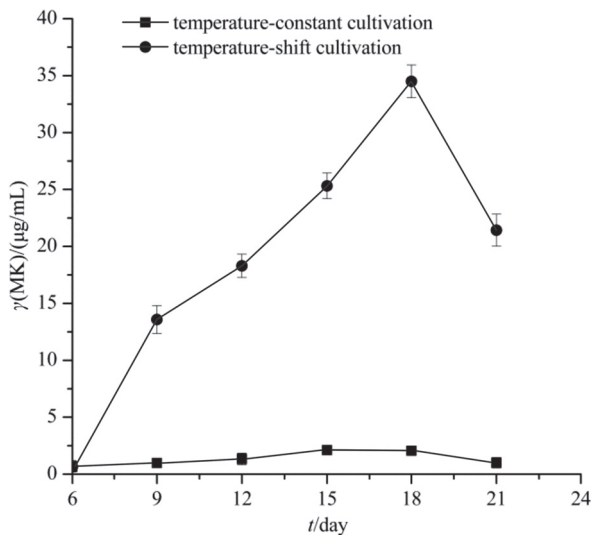
Fig. 1. Cell growth by dry cell mass in temperature-constant (at 30 °C for 21 days) and temperature-shift (at 30 °C for 6 days and at 25 °C for 15 days) cultivations. The data are represented as the mean value±standard error of the mean (SEM) (*N*=3)

Determination of monacolin K content

Monacolin K is a well-known secondary metabolite of *Monascus* sp. because of its ability to lower total cholesterol, low-density lipoprotein cholesterol and triglycerides in the plasma of hyperlipidaemic patients (*17*). Mulder *et al.* (*18*) showed that the culture temperature is an important environmental factor for the increase of MK titers. The linear regression equation of the standard curve was obtained according to the concentration and corresponding peak area and was determined to be:

$$y=63.48x, R^2=0.996 \qquad /1/$$

The different culture temperatures contributed to different concentrations of MK, and the results are shown in Fig. 2. The MK concentration initially increased and then


Fig. 2. Monacolin K (MK) concentration in temperature-constant (at 30 °C for 21 days) and temperature-shift (at 30 °C for 6 days and at 25 °C for 15 days) cultivations. The data are represented as the mean value±SEM (*N*=3)

decreased during both cultivation types at both temperatures. In temperature-constant cultivation it was much lower than that in temperature-shift cultivation. MK was hardly produced in temperature-constant cultivation at 30 °C. The highest concentration of MK in temperature-shift cultivation was observed on the 18th day, whereas that in temperature-constant cultivation occurred on the 15th day. Compared with the highest concentration of MK in temperature-constant cultivation at 30 °C (2.11 µg/mL), the one in temperature-shift cultivation (34.5 µg/mL) was 16 times higher. This result was in agreement with the findings of Tsukahara *et al.* (*5*), who reported that MK was hardly produced in temperature-constant cultivation at 30 °C, but efficient production was observed after lowering the temperature from 30 to 25 °C.

Expression of monacolin K biosynthetic genes

To study the effect of temperature on MK production, the MK biosynthetic gene cluster was selected for evaluating the gene expression level by RT-qPCR (Fig. 3). Gene expression of mycelia cultured at 25 °C was higher than of those cultured at 30 °C, which showed a similar variation trend compared with Fig. 2, except for *MK B* and *MK C* genes. The 2-methylbutyryl-CoA and monacolin J are the precursors for the biosynthesis of MK. At high concentrations they can stimulate its production by promoting *MK F* expression, which encodes transesterase, but also cause feedback inhibition of *MK B* and *MK C* genes. Therefore, the expression levels of *MK B* and *MK C* genes were lower than of those cultured at 30 °C. The biosynthetic pathway of MK was described by Manzoni and Rollini (*19*). The expression of the MK biosynthetic gene cluster increased in parallel with the concentration of produced MK. Notably, the expression of the *MK I* gene in temperature-shift cultivation was 1.65 times higher than that in temperature-constant cultivation, which exhibited
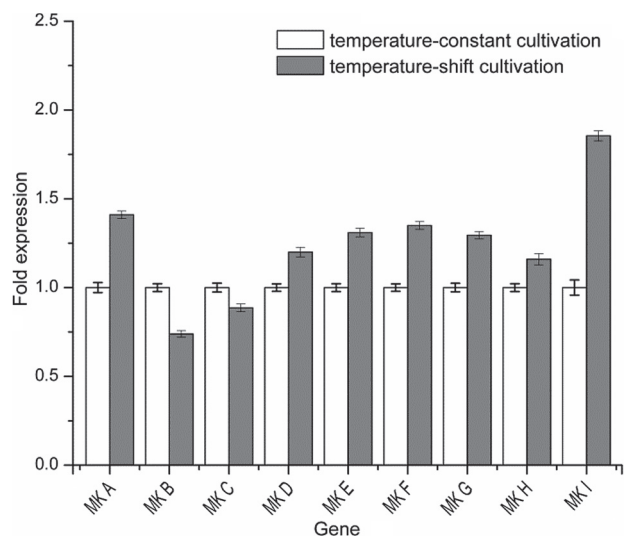

Fig. 3. Expression of monacolin K (MK) biosynthetic genes in temperature-constant and temperature-shift cultivations. All values were normalised using the expression level of the β-actin gene as a reference and the expression level of mycelia cultivated at 30 °C as a calibrator. Data were expressed as the relative mRNA level for each gene and represented as the mean value±SEM (*N*=3)

the greatest change in the MK biosynthetic gene cluster (Fig. 3). This result indicated that temperature-shift cultivation could improve MK production by enhancing the expression of the MK biosynthetic gene cluster. The cultured environment can regulate the production of secondary metabolites by regulating metabolite biosynthesis in the gene cluster; for example, blue light stimulates citrinin production by upregulating the expression of citrinin biosynthesis genes (*20*). Therefore, a low culture temperature (25 °C) could enhance the expression of the MK biosynthesis gene cluster, which finally led to the increase in MK production. A low temperature (25 °C) could enhance the expression of the protein that plays a role in the secondary metabolite production pathway, and suppress the expression of the protein that maintained mycelial growth.

The differential protein expression of mycelia at different culture temperatures

A total of 2016 proteins were detected by LC-ESI-MS/MS, and the protein content of the *Monascus fuliginosus* CG-6 mycelia cultivated at 30 °C was used as the control group. The cut-off of twofold variation was used as the
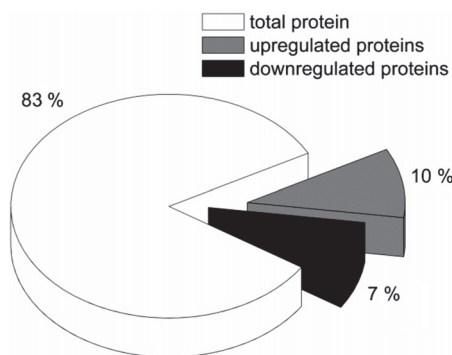
Fig. 4. Pie chart of protein expression changes. Protein content of *Monascus fuliginosus* CG-6 mycelia cultured at 30 °C was used as the control group and cut-off of 2-fold variation was used as judgment index

judgment index, and the results showed 240 upregulated proteins and 180 downregulated proteins. The pie chart of protein expression changes is shown in Fig. 4. The upregulated proteins accounted for 10 % of the total protein, whereas the downregulated proteins amounted to 7 % of the total protein.

A portion of the upregulated proteins is listed in Table 2 (*21*). Among these identified proteins, the evm.model.C4.388 drew attention primarily because it was the efflux pump protein coded by the *MK I* gene with UniProt protein ID of Q3S2U5 (*6*). The expression level of efflux pump proteins in temperature-shift cultivation was 1.35 times higher than that in temperature-constant cultivation (Table 2). Chiang *et al.* (*22*) showed that efflux pump proteins can transport polyketide out of the cell. The expression level of efflux pump proteins exhibited the same trend as the *MK I* gene. Therefore, temperature-shift cultivation could enhance the expression levels of the *MK I* gene and efflux pump proteins, leading to the transport of polyketide MK out of the cell.

In addition to efflux pump proteins, several upregulated proteins play well-known functional roles in metabolites produced by microorganisms. Such metabolites include evm.model.C3.887, which is an ent-kaurene oxidase. Ent-kaurene oxidase can catalyse the oxidation reaction from ent-kaurene to ent-kaurenoic acid, which is the intermediate of gibberellin biosynthesis (*23*). Gibberellin is a phytohormone that can remove the growth limitation of plants, and it is a secondary metabolite of certain fungal species such as *Gibberella fujikuroi*, *Penicillium resedanum* and *Fusarium* sp. (*24,25*). Evm.model.C8.204, described as pyruvate decarboxylase, is a key enzyme responsible for ethanol formation. It can convert the central metabolite pyruvate to acetaldehyde, and alcohol dehydrogenase can reversibly convert acetaldehyde to ethanol (*26*). It exists extensively in microorganisms such as *Rhizopus oryzae*, *Gluconacetobacter diazotrophicus* and *Saccharomyces cerevisiae* (*27,28*). Pyruvate decarboxylase can accelerate metabolite ethanol production.

γ-Amino butyric acid (GABA) is a major inhibitory neurotransmitter of the mammalian central nervous sys-

Table 2. Significantly upregulated proteins of *Monascus fuliginosus* CG-6 mycelia cultivated at 25 °C identified by LC-ESI-MS/MS

Protein ID	No. of matched peptides	Ratio of protein expression levels (25 °C/30 °C)	Gene ontology (*21*)	Function description
evm.model.C2.326	3	30.96	GO:0018342	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha
evm.model.C3.921	4	30.82	GO:0055114	L-arabinitol 4-dehydrogenase
evm.model.C2.668	7	24.93	GO:0006464	Probable E3 ubiquitin-protein ligase hulA
evm.model.C3.887	1	23.13	GO:0020037	Ent-kaurene oxidase
evm.model.C8.204	2	17.48	GO:0030976	Pyruvate decarboxylase
evm.model.C1.159	3	17.18	GO:0055114	Quinone oxidoreductase-like protein 2 homologue
evm.model.C1.548	4	13.02	GO:0006221	CTP synthase
evm.model.C5.371	4	12.99	GO:0008152	4-coumarate-CoA ligase-like 1
evm.model.C6.424	2	7.28	GO:0019752	Glutamate decarboxylase
evm.model.C4.388	1	1.35	GO:0016021	Efflux pump

The upregulated proteins were more than 2-fold compared to the mycelia cultivated at 30 °C, except for evm.model.C4.388

Table 3. Significantly downregulated proteins of *Monascus fuliginosus* CG-6 mycelia cultivated at 25 °C identified by LC-ESI-MS/MS

Protein ID	No. of matched peptides	Ratio of protein expression levels (25 °C/30 °C)	Gene ontology (21)	Function description
evm.model.C4.455	4	0.023	GO:0006508	Mitochondrial-processing peptidase subunit alpha
evm.model.C6.692	2	0.034	GO:0006486	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit stt3
evm.model.C4.541	3	0.039	GO:0008152	Peroxisomal 3-ketoacyl-CoA thiolase
evm.model.C2.114	2	0.056	GO:0008152	Tyrosinase
evm.model.C5.746	3	0.083	GO:0008152	Mitochondrial 2-methylisocitrate lyase
evm.model.C7.460	7	0.11	GO:0046488	Phosphatidylinositol 4-phosphate 5-kinase its3
evm.model.C4.802	3	0.15	GO:0055114	Putative acyl-CoA dehydrogenase AidB
evm.model.C3.836	2	0.16	GO:0055085	ATP-dependent bile acid permease
evm.model.C7.89	3	0.16	GO:0006071	Glycerophosphodiester phosphodiesterase GDE1
evm.model.C4.176	4	0.30	GO:0006468	cAMP-dependent protein kinase type 2

The downregulated proteins were less than 0.5-fold compared to the mycelia cultivated at 30 °C

tem. The glutamate decarboxylase (GAD), which has a protein ID evm.model.C6.424 in this study, is the main enzyme for producing GABA. GAD is an intracellular enzyme that is commonly used as a major catalyst in the trial biotransformation of GABA (29). GABA can be produced by many kinds of organisms such as plants and fungi (*Trichoderma atroviride*, *Aspergillus oryzae* and *Monascus* sp.) (30–32). The upregulated proteins play various roles, especially in secondary metabolite production.

Certain downregulated proteins (less than 0.5-fold compared with mycelia cultivated at 30 °C) are listed in Table 3. Among these proteins, several have well-known functions in growth metabolism. The protein with the ID evm.model.C5.746 was described as 2-methylisocitrate lyase in mitochondria. The 2-methylisocitrate lyase is a subfamily of the isocitrate lyase superfamily, which can catalyse the cleavage of isocitrate to glyoxylate and succinate. The glyoxylate pathway is important for the growth of microorganisms on fatty acids, acetate, ethanol and amino acids (33). The glyoxylate cycle and isocitrate lyase are widely distributed among archaea, bacteria and fungi (34,35). Evm.model.C4.802 was described as acyl-CoA dehydrogenase (ACAD), which can catalyse the α,β-dehydrogenation of acyl-CoA esters in fatty acid and amino acid catabolism. The ACADs are mitochondrial flavoenzymes involved in fatty acid and amino acid catabolism, and they can produce ATP to aid organism growth (36). The function description of evm.model.C4.176 is cAMP-dependent protein kinase A (PKA), which can mediate the cAMP reaction in eukaryotic cells (37). PKA belongs to the serine-threonine protein kinase superfamily and participates in the control of various cellular processes (38). Among the downregulated proteins, several proteins act on growth metabolism.

Conclusions

In this study, *Monascus fuliginosus* CG-6 was cultured under temperature-constant (30 °C for 21 days) and temperature-shift (30 °C for 6 days and 25 °C for 15 days) conditions. The mycelial mass and monacolin K (MK) concentration at different culture temperatures were measured. Results showed that temperature-constant cultivation at 30 °C could enhance the *Monascus fuliginosus* CG-6 dry cell mass growth, but the MK concentration was 16 times lower than that during temperature-shift cultivation.

This study is the first to relate the expression of MK biosynthetic genes with MK production in *Monascus* sp. Gene expression analysis showed that gene expression of mycelia cultivated at 25 °C was higher than that at 30 °C, which was similar to the trend observed with the MK concentration, except for *MK B* and *MK C* genes. The results also revealed that the expression of the *MK I* gene during temperature-shift cultivation was 1.65 times higher than that during temperature-constant cultivation. The total protein of mycelia under two different temperatures was collected and analysed by LC-ESI-MS/MS. The efflux pump protein coded by the *MK I* gene was found in the upregulated proteins, which demonstrated the same upregulation trend with the *MK I* gene. However, the other proteins coded by the other genes of the MK biosynthesis gene cluster could not be measured because only one complete genome sequence of *Monascus* sp. exists. Among the upregulated proteins, we found that several proteins played various important roles, especially in secondary metabolite production, such as ent-kaurene oxidase, pyruvate decarboxylase and GAD. Several downregulated proteins act on growth metabolism, such as 2-methylisocitrate lyase, ACADs and PKAs, which play essential roles in the growth of microorganisms. The results of protein expression corresponded with the dry cell mass growth and MK concentrations, which suggested that a low temperature (25 °C) stimulated the organism to produce secondary metabolites, but a high temperature (30 °C) promoted mycelial growth.

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