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 biosynthesis. MK can inhibit cholesterol biosynthesis and lower blood cholesterol levels in both humans and animals (2). Therefore, it is known as an anticholesterol and antihypertensive 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
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 cultivation temperature to 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 mechanisms 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, KH2PO4 1, NaNO3 1 and MgSO4·7H2O 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 4 days, followed by additional 17 days.

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 cultures was determined by removing the cellophane to recover the mycelia of the two groups. The biomass concentration was approx. 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 synthesized by PrimeScript 1st strand cDNA synthesis kit (Taka rara 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. DQ176951.1) (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>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Product size/bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin-F</td>
<td>AGTCCAACAGGGAAGACTGT</td>
<td>132</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>CACCCAGTCAGACCGACGATA</td>
<td></td>
</tr>
<tr>
<td>MK A-F</td>
<td>GAACCATCGCCGCGCAAT</td>
<td>179</td>
</tr>
<tr>
<td>MK A-R</td>
<td>ACAAAGGTCGCAATGCACTCAG</td>
<td></td>
</tr>
<tr>
<td>MK B-F</td>
<td>AAGTGATGAGGGAGGTGTAAG</td>
<td>159</td>
</tr>
<tr>
<td>MK B-R</td>
<td>AATGGAAGGACCTGTAGT</td>
<td></td>
</tr>
<tr>
<td>MK C-F</td>
<td>TGGCCGGAAAGAATGCACC</td>
<td>152</td>
</tr>
<tr>
<td>MK C-R</td>
<td>CTTTGAGGCGAGAAGAACC</td>
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<tr>
<td>MK D-F</td>
<td>ATGGGGGAGGGAGAGTC</td>
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</tr>
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<tr>
<td>MK E-F</td>
<td>ATCCCTCAGGCGGTGC</td>
<td>194</td>
</tr>
<tr>
<td>MK E-R</td>
<td>GCCGCTATGTCCTCCGTGAAC</td>
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</tr>
<tr>
<td>MK F-F</td>
<td>AAAAGGGAAGACAGATGAC</td>
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</tr>
<tr>
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</tr>
<tr>
<td>MK G-F</td>
<td>GACGCCGGACAGTGTAAG</td>
<td>161</td>
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<td>CGAGCCCGAATACAAAAAG</td>
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<tr>
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<td>CAGAACCCCAAACACCCAC</td>
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</tr>
<tr>
<td>MK I-R</td>
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<td></td>
</tr>
</tbody>
</table>
(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^−ΔΔCq method (9). All values were normalised using the reference expression level of the β-actin gene. The 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 using the Lowry method (12). Brieﬂy, the protein N-terminal were speciﬁed as variable 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 set 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 quantitation 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.

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:

\[ y = 63.48x, \quad R^2 = 0.996 \]

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 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
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 biosynthetic 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 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 Q8S2U5 (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 Saccaromyces 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-

![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.](image)

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>No. of matched peptides</th>
<th>Ratio of protein expression levels (25 °C/30 °C)</th>
<th>Gene ontology (21)</th>
<th>Function description</th>
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<tbody>
<tr>
<td>evm.model.C2.326</td>
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<td>30.96</td>
<td>GO:0018342</td>
<td>Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha</td>
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<tr>
<td>evm.model.C3.921</td>
<td>4</td>
<td>30.82</td>
<td>GO:0035114</td>
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<tr>
<td>evm.model.C2.668</td>
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<td>24.93</td>
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<td>Probable E3 ubiquitin-protein ligase hulA</td>
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<td>3.53</td>
<td>GO:0016021</td>
<td>Efflux pump</td>
</tr>
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</table>

The upregulated proteins were more than 2-fold compared to the mycelia cultivated at 30 °C, except for evm.model.C4.388.
acid catabolism. The ACADs are mitochondrial generation of acyl-CoA esters in fa...tus functions in growth metabolism.

Table 3. Among these proteins, several have well-known roles, especially in secondary metabolite production.

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

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 protein 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 the aMP-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.

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References


