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Full Length Article

Isolation, Screening and Fermentation Optimization of *Monascus* Strains with High Monacolin K Yield and the Cholesterol Lowering Effect of Red Yeast Rice

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Abstract

Monacolin K (MK), one of the main active ingredients of red yeast rice, has cholesterol-lowering effect. This study aimed to improve MK content in red yeast rice through three aspects including strain and rice material screening and optimization of fermentation conditions. The cholesterol-lowering effect of high MK red yeast rice in mice was also studied. The results showed that: *Monascus* strain FG-2 has the highest MK yields, about 2.3 times of model strain ACCC 30501. Molecular identification reveals the strain as *Monascus purpureus*. When fermented with polished rice of variety "1a825" as substrate, strain FG-2 and the highest MK yield, about 2.7 times of commercially available rice "Dulimi". The optimum fermentation conditions for strain FG-2 were determined by single factor optimization and orthogonal test: pre-fermentation temperature at 30°C, seed solution pH at 5.0, rice amount in fermentation flask 40 g, fermentation time 17 d. MK yield under this fermentation condition was 4.64 mg/g, 28.5% higher than before optimization, which was about 8.3 times of commercially available Gutian red yeast rice. The high MK red yeast rice prepared herein and commercially available Gutian red yeast rice have significant cholesterol lowering effects, with the former superior to the latter.

Keywords: *Monascus*; Monacolin K; Strain screening; Rice raw material; Fermentation condition; Cholesterol lowering

Introduction

Red yeast rice, described as a traditional food and medicine in the Chinese Pharmacopoeia, originated in China thousands of years ago (Erdoğrul and Azirak, 2004). As purple-red rice koji made from rice as raw material and fermented by *Monascus*, red yeast rice is a natural food additive benefiting human health (Zlová *et al*., 1996).

Endo (1979) first extracted an active substance that inhibits cholesterol synthesis in the body from culture of *Monascus ruber* and named it as Monacolin K (MK, also known as lovastatin, mevastatin). Alberts *et al*. (1980) extracted lovastatin from the culture of *Aspergillus terreus*, which has the same substance as MK. MK, an active ingredient in red yeast rice, lowers blood cholesterol (Barrios-Gonzalez *et al*., 2008), antibiosis (Zhou *et al*., 2018), colon cancer (Hong *et al*., 2008), breast cancer (Zhu *et al*., 2012), anti-oxidation (Dhale *et al*., 2007; Awad *et al*., 2017), lowering prostate (Shannon *et al*., 2005), liver protection (Wei and Popovich, 2013) and accelerating nerve regeneration (Ghayour *et al*., 2017). MK can effectively reduce cholesterol in dogs (Alberts *et al*., 1980), reduce fibrinogen content in blood of high-fat model mice, increase antithrombin III and protein C levels, and reduce aortic lipid plaques (Lee *et al*., 2013). In clinical trials, MK significantly reduced total cholesterol and low-density lipoprotein cholesterol levels, suggesting that MK also has lipid-lowering effect on humans (Ruscica *et al*., 2014). MK has been widely used as an effective drug to lower blood cholesterol (Panda *et al*., 2010), arousing the attention to red yeast rice in medical field. Therefore, how to improve MK content in red yeast rice is still a research hotspot in the field

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of hypolipidemic drugs.

MK has a highly effective and specific inhibitory effect on rate-limiting enzyme HMG-CoA reductase (HMGR) of the cholesterol synthesis pathway, thereby lowering cholesterol synthesis (Singgih *et al*., 2014). Natural MK has two different structures, namely open loop β-hydroxy acid form and closed-loop lactone form (Kennedy *et al*., 1999; Huang *et al*., 2010). MK mainly exists in an ester-closed loop structure in naturally fermented red yeast rice, but only MK in open-loop acid structure can bind with HMGR to exert an inhibitory effect. Gum *et al*. (2017) found that red yeast rice fermented with *Bacillus subtilis* significantly increased acid ester proportion in lovastatin and antioxidant activity (Gum *et al*., 2017).

The study on the biosynthesis pathway of MK and lovastatin began with *A. terreus*. In 1999, there were reports on the genes related to lovastatin synthesis in *A. terreus* (Kennedy *et al*., 1999) and the synthetic pathway of lovastatin in *A. terreus* has been well studied (Guo and Wang, 2014; Yin *et al*., 2016). Later, the genes of MK synthesis in *Monascus* were also studied. MK is a secondary metabolite synthesized by polyketide synthase (PKS) in *Monascus*. Chen *et al*. (2008) cloned the genome sequence of MK biosynthetic gene cluster from *Monascus pilosus* (GenBank: DQ176595.1), composed of 9 genes *mok A* - *mok I*, which has similarity of over 54% with the lovastatin biosynthetic gene cluster from *A. terreus*. Then, the core of *mok A* in the wild-type *Monascus* pilosus strain was replaced through homologous recombination, forming strain lacking *mok A*. Such strain did not produce MK, indicating that *mok A* encodes polyketide synthase responsible for MK synthesis in *Monascus pilosus* (Chen *et al*., 2008). The *mok B* is located at the end of the gene cluster–and knockout strain failed to produce MK, but accumulated the intermediate product of MK synthesis pathway, Monacolin J (MJ), indicating that *mok B* encodes polyketide synthase and is responsible for MK side chain diketone synthesis (Sakai *et al*., 2009). The *mok H* encodes Zn (II)2 cys6 dinuclear DNA binding protein as a catalyst for MK synthesis. The *mok H* can play a positive regulatory role in the transcription of MK synthesis-related genes, thereby increasing MK yield (Chen *et al*., 2010). Lin *et al*. (2018) overexpressed *mok E* in *Monascus fulginosus* and showed that *mok E* expression level of the transformed strain was 4.8 times higher than the wild type strain and MK yield was increased by 2.5 times. In recent years, high-throughput sequencing technology has also been applied to the study of lovastatin and MK synthesis related genes. Savitha *et al*. (2016) reported whole genome sequence of *A. terreus* (KM017963), including complete sequence of lovastatin biosynthesis gene cluster. Zhang *et al*. (2017) performed de novo RNA sequencing and transcriptome analysis on *Monascus purpureus* cultured for different days, demonstrating that *mok F* plays a regulatory role in MK synthesis, and 9 genes (*mok A* – *mok I*) are related to MK synthesis.

In addition to association with the genotype of microorganisms, the production of microbial secondary metabolites is largely influenced by environmental conditions. Studies have shown that *A. terreus* uses glycerol and lactose as carbon sources, which is more conducive to the synthesis of lovastatin. However, when only glycerol is used as a carbon source, geodin will be preferably synthesized (Hasan *et al*., 2018). This is consistent in *Monascus*. The carbon and nitrogen source types, pH, culture temperature and light of the culture medium will all affect its MK yield (Calvo *et al*., 2002; Li *et al*., 2010). Lin *et al*. (2017) studied the effect of culture temperature on the yield of *Monascus fulginosus* MK, finding that constant temperature culture could promote mycelial growth of *Monascus*, but did not facilitate MK production. However, under the condition of varying temperature, *Monascus* MK synthesis related genes had expression level higher than those of constant temperature culture, with MK yield 16 times higher than constant temperature culture.

There are mainly two ways to increase MK yield of *Monascus*. One is to select the strain with high MK yield, and the other is to screen the optimum fermentation conditions for MK production, such as culture temperature, pH, inoculum size, stirring/ventilation condition, fermentation time, carbon nitrogen ratio (Mulder *et al*., 2015). In the liquid fermentation process, hyphae tend to accumulate into mycelium pellet and appropriate volume of mycelium pellet promotes MK formation. However, as the fermentation proceeds, the mycelium pellet increases volume and fermentation liquid viscosity, hindering the transportation of nutrients and oxygen so that the interior of the mycelium pellet cannot be replenished in time, causing autolysis and "hollowness" of the interior mycelium pellet, which in turn affects MK generation. Through stirring, the hyphae can be prevented from being too dense, the mycelium pellet volume can be reduced, and the dissolved oxygen can be increased to improve the transportation of nutrients and oxygen in the liquid (Mulder *et al*., 2015). Studies have shown that red yeast rice produced by solid state fermentation has higher MK yield than liquid fermentation (Zhang *et al*., 2013, 2015). At present, some studies focus on how to improve MK content in red yeast rice, mainly by transforming the strain, such as: engineering its key genes by molecular means, or performing mutagenesis by physical, chemical methods (Mulder *et al*., 2015) and even metabolically engineering fungus that could perform de novo synthesis of MJ and MK with methanol as a precursor (Liu *et al*., 2018). However, there are rare reports on the differences in MK production by *Monascus* fermentation using different varieties of rice materials. In this study, the optimal combination of MK high-yield strains and rice raw materials was screened, fermentation process in MK production was optimized, cholesterol-lowering effect of red yeast rice was studied via mice test to provide experimental basis for preparation and application of high MK red yeast.

Materials and Methods

Experimental Materials

Red yeast powder: 7 kinds in total were purchased from Hebei, Beijing, Gansu, Guangdong and Fujian Province in China.
***Monascus* strains**: 13 strains in total were purchased from Agricultural Culture Collection of China (ACCC), China Center of Industrial Culture Collection (CICC) and Guangdong Microbiology Culture Center (GDMCC) in China.
Rice varieties: a total of 26 varieties were obtained from Key Laboratory of Crop Biotechnology, Fujian Agriculture and Forestry University, Fujian Province University, China.
Test animals: KM strain male mice (clean grade, weight 15–20 g/mice), were purchased from Laboratory Animal Center, Fujian Medical University in China.

Rice Planting and Rice Raw Material Preparation

All the rice varieties were planted in the field of Fujian Agriculture and Forestry University, in Minhou, Fuzhou. The field management was conducted the same as common rice varieties. After the maturation of rice grain, three samples were randomly taken from each variety. The samples were then grained to milled rice and brown rice for later use. The market bought common rice "Dulimi" and "Gutian red yeast rice" were used as controls.

Separation of *Monascus* Strains

The 5 g of commercially available red yeast powder was transferred into a 250 mL flask containing 45 mL sterile physiological saline, and shaken at 150 rpm for 30 min to prepare diluents with a concentration gradient of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The diluents were spread on PDA medium plates and incubated at 28°C for 3–5 d. All single colonies with *Monascus* characteristics were selected and arranged in a "Z" shape on a new PDA plate, cultured at 28°C for 3–5 d and transferred to a new PDA plate. After multiple isolation and purification, it was transferred to PDA slant medium, cultured at 28°C for 5 d, and stored in a 4°C refrigerator for later use.

Preparation and Inoculation of Culture Medium

Preparation of seed solution medium: glucose 50 g, peptone 5 g, yeast extract 1 g, KH_2PO_4 1 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $MgSO_4 \cdot 7H_2O$ 0.5 g. Add water to a constant volume of 1000 mL. Each 50 mL of seed culture medium was dispensed into a 250 mL flask, added with eight glass beads and autoclaved at 121°C for 20 min.

Preparation of rice fermentation medium: Wash the rice, soak in water for three hours, steam for 20 min, then each 50 g of rice was dispensed into 300 mL plastic fermentation bottle and autoclaved at 121°C for 20 min.

After the *Monascus* strains were activated and cultured on the PDA plate, the fungus cake was taken with a 5 mm diameter puncher and 5 pieces of the cake were added to a 250 mL flask containing 50 mL of the seed liquid medium (each bottle was put with eight small glass beads) and cultured at 30°C, 150 rpm for 72 h. The seed solution was diluted to a spore concentration of about 1×10^7 cell/mL, added to a fermentation flask containing rice fermentation medium at 10% inoculums size, and cultured in a 28°C incubator for 16 d. There were three repetitions for each treatment.

HPLC Detection of MK Content in Red Yeast Rice

Pretreatment of the sample to be tested: The solid fermentation product was dried at 50°C to constant weight, and ground into powder. The 0.5 g sample to be tested was added to a 10 mL brown volumetric flask and 5 mL of 0.2 mol/L NaOH solution was added and volume was adjusted to 10 mL with methanol and sonicated at 50°C for 1 h. After centrifugation at 8000 g for 10 min at 4°C, 5 mL of supernatant was transferred to the brown volumetric flask and adjusted to 25 mL with methanol. After full shaking, 1 mL of the diluents was taken and passed through 0.45 μm filter to be injected.

HPLC chromatographic conditions: chromatographic column Vision HT C18HL (5 μm, 250 × 4.6 mm), Grace; mobile phase, acetonitrile: 0.18% phosphoric acid solution = 50:50 (v/v); flow rate 1.0 mL /min; detector wavelength 238 nm; column temperature 28°C; injection volume 10 μL.

Molecular Identification of High-yield MK *Monascus* Strains

Genomic DNA of high-yield MK *Monascus* mycelium was extracted using genomic DNA rapid extraction kit (Beijing Ding Guo Changsheng Biotechnology Company, Ltd., China) and its ITS sequence was amplified with 2× *Eco* Taq PCR SupperMix (TransGen Biotech, AS151). The primers were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR reaction system had a total volume of 50 μL containing 25.0 μL 2× *Eco* Taq PCR SuperMix, 1.0 μL each of the forward and reverse primers (20 μM), 4.0 μL DNA template and 19.0 μL ddH_2O. The PCR reaction conditions were 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s and a final extension at 72°C for 10 min. The amplified ITS fragment was recovered and sent to BioSune Biotechnology (Shanghai) Co., Ltd. for sequencing with primers ITS1 and ITS4, and the sequence obtained was aligned with the reported ITS sequence in the GenBank database using BLASTN to obtain species information of the high-yield MK *Monascus* strain.

In vivo Cholesterol-lowering Test of High MK Red Yeast Rice

KM mice were divided into five groups: basal feed control

group A, hyperlipidemia model control group B, Gutian red yeast powder test group C, homemade red yeast powder test group D, Xuezhikang drug test group E, with 10 mice in each group. At the start of the experiment, mice were grouped by weight to make overall weight of each group as balanced as possible. Each group was fed with basal feed every day and was free to drink. Except group A, each other group was intraperitoneally injected with 0.2 mL of 75% fresh egg yolk emulsion for 7 consecutive days to establish experimental hyperlipidemia mouse model. Blood was drawn from the mice tail in each group, centrifuged at 3000 g for 15 min to separate serum after standing for 15 min. The levels of TC and TG in the serum were measured using a total cholesterol (TC) kit and a triglyceride (TG) kit (Nanjing Jian Cheng Biotechnology Company, Ltd., China), and various cholesterol indexes of the initial serum were recorded.

Groups C, D and E were fed daily with feed mixed with equal amounts (0.2 g/group) of Gutian red yeast powder, homemade red yeast powder and Xuezhikang drug respectively for 3 weeks. Groups A and B were fed with basal feed. After the last administration, each group of mice was fasted (without water fasting) for 12 h and then the levels of TC and TG in the serum were determined for each group with reference to the above method.

The experimental data in this study were all statistically analyzed using DPS data processing system.

Results

Separation and Screening Results of High-yield MK *Monascus* Strains

A total of 27 *Monascus* strains with good mycelial growth were isolated and purified from 7 commercially available samples of red yeast powder, and 13 strains were purchased from the Culture Collection. The solid fermentation products of 40 strains were pretreated, with their MK content determined by HPLC. The results showed that the MK yields of strains FG-2, APS 012, FG-1, FG-4, HB-3 and BG-2 were significantly higher than the model strain ACCC 30501. The strain with the highest MK yield was FG-2, reaching 2.46 mg/g, about 2.3 times that of the control strain ACCC 30501.

Molecular Identification Results of High-yield MK *Monascus* Strains

The ITS sequence amplification product of strain FG-2 showed a clear specific band between 500–600 bp (Fig. 1). The results of sequencing and alignment analysis showed that the strain has the highest ITS sequence similarity to the *Monascus purpureus* strain Mp-41, reaching 99% (Fig. 2). Therefore, strain FG-2 was identified as *M. purpureus*.

Effects of Different Rice Raw Materials on MK Yield by *Monascus* Fermentation

The solid fermentation test of strain FG-2 was carried out using 13 varieties of rice as raw materials. Each rice variety was divided into polished and brown rice, with a total of 26 rice raw materials. With the commercially available high-quality rice "Dulimi" as the control CK1 and the commercially available Gutian red yeast rice as the control CK2, comparison was made on the effects of different rice raw materials on MK yield. The results show that rice raw materials in different varieties have significant ($P < 0.01$) effects on MK yield of *Monascus* (Table S1) Glutinous rice was not suitable as rice raw material for *Monascus* fermentation. MK yield was higher when polished rice was used as the raw material compared to brown rice of the same variety. When the rice variety "1a825" (polished rice) was used as raw material, the MK yield was the highest, reaching 3.61 mg/g, about 2.7 times that of "Dulimi" and 6.5 times that of the commercially available Gutian red yeast rice. Therefore, in the subsequent experiments of this study, the rice variety "1a825" (polished rice) was used as the raw material for *Monascus* fermentation.

Single Factor Optimization Results of Solid State Fermentation Conditions

Effect of rice amount in fermentation bottle on MK yield in fermentation products: The 20 g, 30 g, 40 g, 50 g, 60 g of rice steamed until zero white core were weighed and placed in a plastic fermentation bottle. Refer to 1.2.2 for other conditions. The results showed that (Fig. 3): the rice amount of fermentation bottle has a great influence on MK yield. When the rice amount is too small, the medium tends to lose moisture and the nutrients will dry quickly, unable to provide the nutrients necessary for normal *Monascus* growth; when the rice amount is too much, the oxygen in the fermentation bottle is insufficient, which is not conducive to *Monascus* growth and in turn affects MK formation. MK yield was the highest when the rice amount was 30 g, reaching 4.67 mg/g.

Effect of pre-fermentation temperature on MK yield in fermentation products: The red yeast fermentation bottles were placed in an incubator at 26°C, 28°C, 30°C and 32°C, respectively. On the 8th day, all were transferred to a 26°C incubator for further fermentation. Refer to 1.2.2 for the other conditions. The results showed that (Fig. 4): pre-fermentation temperature also has a great influence on MK yield. Temperature can directly affect the growth rate of mycelium. When the pre-fermentation temperature is too low, the mycelium grows slowly and the amount of fermentation products decreases. When the pre-fermentation temperature is too high, the mycelium is too exuberant with nutrients consumed in advance, which is also not beneficial to the accumulation of fermentation products in the later

Table S1: MK Content in red yeast rice fermented by different *Monascus* strains

Strain number	MK yield (mg/g)	significance level of difference ($P < 0.05$)	significance level of difference ($P < 0.01$)
FG-2	2.46±0.21	a	A
CICC 5037	1.68±0.15	b	B
FG-1	1.48±0.12	bc	BC
FG-4	1.26±0.11	cd	BCD
HB-3	1.19±0.10	cde	CDE
BG-2	1.17±0.09	cdef	CDE
ACCC 30501 (CK)	1.08±0.08	defg	CDEF
FG-3	1.07±0.09	defg	CDEF
BG-1	0.97±0.08	defgh	DEFG
CICC 5004	0.86±0.07	efghi	DEFGH
CICC 5026	0.86±0.08	efghi	DEFGH
ZA-2	0.86±0.07	efghi	DEFGH
GD-2	0.82±0.07	fghij	DEFGH
AX-2	0.78±0.06	ghijk	DEFGHI
GS-1	0.76±0.06	ghijk	EFGHI
GS-2	0.73±0.06	ghijk	EFGHIJ
GD-3	0.68±0.05	hijkl	FGHIJK
CICC 5022	0.66±0.06	hijklm	FGHIJKL
GD-1	0.64±0.05	hijklm	FGHIJKL
CICC 5016	0.55±0.04	ijklmn	GHIJKLM
CICC 5027	0.53±0.04	ijklmno	GHIJKLM
FG-5	0.50±0.05	ijklmnop	GHIJKLM
ACCC 30352	0.50±0.04	ijklmnop	GHIJKLM
ZA-3	0.49±0.04	ijklmnop	GHIJKLM
JN-1	0.46±0.03	jklmnopq	HIJKLM
CICC 5009	0.44±0.03	klmnopq	HIJKLM
GD-5	0.43±0.04	klmnopqr	HIJKLM
GD-4	0.43±0.03	klmnopqr	HIJKLM
CICC 5001	0.32±0.02	lmnopqr	IJKLM
GIM 3.239	0.32±0.02	lmnopqr	IJKLM
HB-2	0.32±0.02	lmnopqr	IJKLM
GS-4	0.31±0.03	mnopqr	IJKLM
FX-1	0.25±0.02	nopqr	JKLM
ACCC 30342	0.23±0.02	nopqr	KLM
FX-2	0.21±0.02	nopqr	KLM
FX-3	0.17±0.01	opqr	LM
GS-3	0.15±0.01	pqr	M
CICC 5025	0.14±0.01	pqr	M
FG-6	0.08±0.01	qr	M
AX-1	0.07±0.01	r	M

Different letters in the same column of 'significance level of difference' indicate significant differences ($P < 0.05$ or $P < 0.01$), the same below.

stage. Therefore, in the early stage of fermentation (1–7 d), it is appropriate for mycelium to grow at a higher temperature; and the later (after 8 d) low temperature environment is helpful to the formation of secondary metabolites. MK yield was the highest when the pre-fermentation temperature was 30°C, reaching 4.30 mg/g.

Effect of seed solution pH on MK yield in fermentation products: Five pH levels of the seed solution were selected: 4.0, 4.5, 5.0, 5.5 and 6.0. Refer to 1.2.2 for other conditions. The results showed (Fig. 5) that seed solution pH had a significant effect on MK yield. As *Monascus* is acidophilic and suitable for growth in an acidic environment, seed solution pH can be adjusted with acetic acid to increase MK

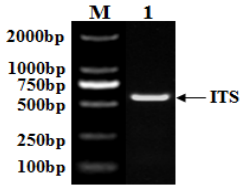

Fig. 1: Amplification of ITS sequence from *Monascus* strain FG-2. (M) DNA Marker DL 2000. (1) amplification of ITS from *Monascus* strain FG-2

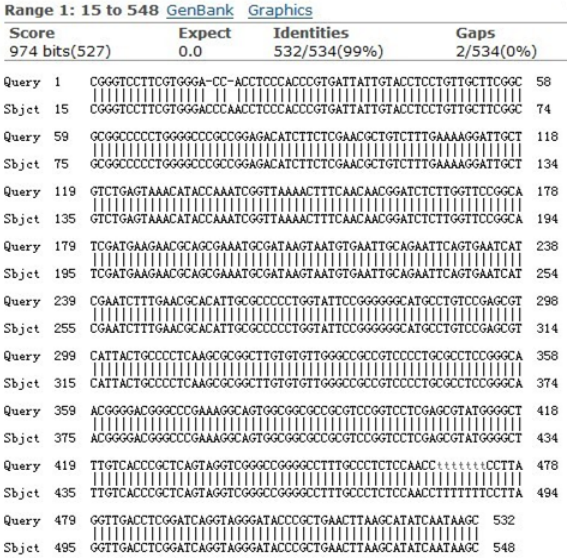

Fig. 2: Alignments of ITS sequence from *Monascus* strain FG-2 with the reference sequence from *M. purpureus* strain Mp-41

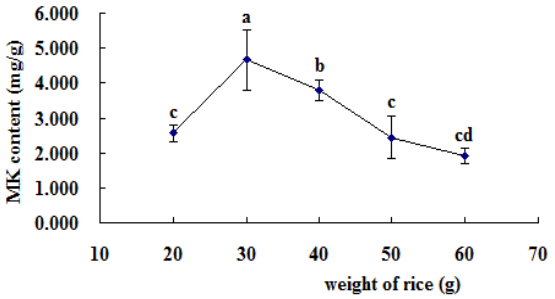

Fig. 3: Effects of rice weight loaded in a fermentation flask on the production of MK. Different letters in different treatment indicate significant differences ($P < 0.05$), the same below

yield. When seed solution pH is below 5.0, MK yield increases with pH increase; when the seed solution pH is over 5.0, MK yield decreases significantly with pH increase. MK yield was the highest when seed solution pH was 5.0, reaching 4.18 mg/g.

Effect of *Monascus* inoculum concentration on MK yield in fermentation products: Five inoculum sizes were selected: 6, 8, 10, 12 and 14% (v/m). Refer to 1.2.2 for other

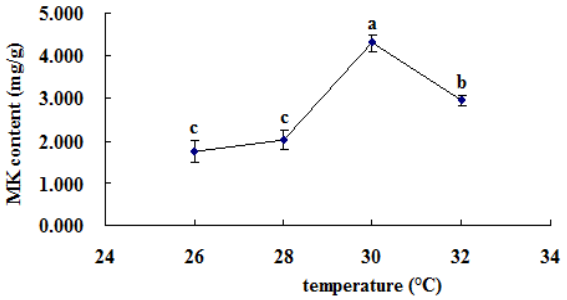

Fig. 4: Effects of temperature of fermentation prophase on the production of MK

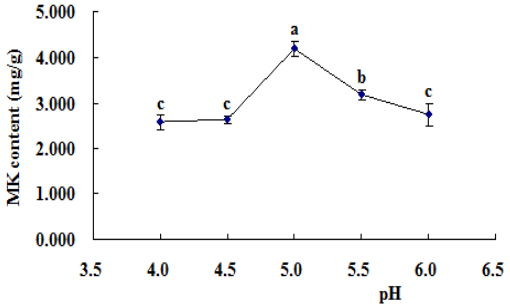

Fig. 5: Effects of pH of seed liquid on the production of MK

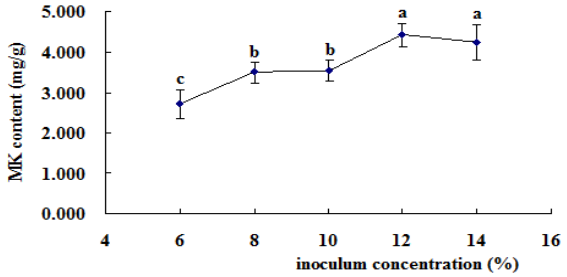

Fig. 6: Effects of inoculum concentration of *Monascus* on the production of MK

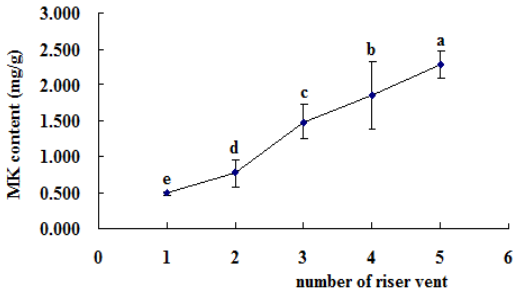

Fig. 7: Effects of ventilation property of fermentation flask on the production of MK

conditions. The results showed that (Fig. 6): the inoculums size of seed solution had a significant effect on MK yield. When the inoculums size is too small, with excessive culture medium nutrient, slowed mycelium growth and prolonged fermentation cycle, MK yield is low; when the inoculums size is excessive, with exuberant mycelium growth and insufficient supply of medium nutrient and oxygen, MK yield is limited. MK yield was the highest when the inoculum size was 12%, reaching 4.42 mg/g.

Effect of fermentation bottle permeability on the yield of MK in fermentation products: The fermentation bottle permeability was adjusted by changing the number of air holes of the fermentation bottle. Five air hole levels of 1, 2, 3, 4 and 5 were selected. Refer to 1.2.2 for other conditions. The results showed that (Fig. 7): fermentation bottle permeability has a great influence on MK yield. MK yield increases with the gas permeability increase. *Monascus* is an aerobic microorganism, which has a high oxygen demand in the fermentation process. Better fermentation bottle permeability is favorable for the growth and fermentation of *Monascus* under the premise of no contamination by infectious microbe, leading to increased MK yield. Accordingly, MK yield of *Monascus* can be increased by appropriately increasing fermentation bottle permeability.

Effect of fermentation time on MK yield in fermentation products: During the fermentation, samples were taken at 5 d, 8 d, 11 d, 14 d, 17 d and 20 d respectively to determine MK content. The results show that (Fig. 8): MK is not detected in the fermentation products at the initial fermentation stage (⩽5 d), indicating that *Monascus* is still in the process of mycelium growth in this stage, and no MK is formed; in the fermentation metaphase (8–17 d), mycelium growth is basically completed, MK gradually forms; when fermented to 17 d, MK yield is the highest; when fermented to 20 d, MK content decreases. During the fermentation of *Monascus* strain FG-2, MK yield reached its maximum at 17 d.

Effect of additional carbon source types on MK yield in fermentation products: Glucose, sucrose, α-lactose and soluble starch were added to the rice fermentation medium as additional carbon sources. The addition amount was 3% (w/w) and that without additional carbon source was taken as the control group. Refer to 1.2.2 for other conditions. The results show that (Fig. 9): addition of soluble starch and glucose can significantly increase MK yield of the fermentation product, while the addition of sucrose and α-lactase has no significant effect on MK yield. Soluble starch is more expensive than glucose, so glucose can be selected as an additional carbon source to increase MK yield in consideration of the production cost.

Effect of additional nitrogen source types on MK yield in fermentation products: Yeast extract, peptone, sodium nitrate and ammonium sulfate were added to the rice fermentation medium as additional nitrogen sources. The addition amount was 4% (w/w) and that without additional nitrogen source was taken as the control group. Refer to 1.2.2 for other conditions. The results show that (Fig. 10): addition of sodium nitrate can greatly increase MK yield, followed by the effect of peptone and ammonium sulfate, and yeast has least effect. Therefore, sodium nitrate can be selected as an additional nitrogen source to increase MK yield.

Effect of rice soaking time on MK yield in fermentation products: Five rice soaking time was selected: 0 h, 3 h, 6 h, 9 h and 12 h. The rice was drained after soaking. Refer to 1.2.2 for other conditions. The results show that (Fig. 11): MK yield gradually decreases with the increase of rice soaking time. It is possible that the rice variety "1a825" is suitable for direct cooking without soaking. Hence, the rice is directly cooked after cleaning, which is more favorable for MK production in *Monascus* fermentation.

Effect of Additional Precursor Substance on MK Yield in Fermentation Products: It can be known from the synthetic pathway of MK that, acetic acid is a precursor substance for MK synthesis, but acetic acid is volatile and has some toxicity to the thallus growth. Therefore, sodium acetate is usually used instead of acetic acid as a precursor substance of MK (Mulder *et al*., 2015).

The 1% (w/w) sodium acetate was added to the rice fermentation medium and that without sodium acetate was taken as the control group. Refer to 1.2.2 for other conditions. The results show (Fig. 12) that addition of 1% sodium acetate can increase MK yield by 44.8%.

Orthogonal Optimization Results of Solid State Fermentation Conditions

According to the above single factor optimization experiment results, three levels of the four factors were selected for orthogonal optimization of solid state fermentation conditions of strain FG-2: pre-fermentation temperature (28, 30, 32°C), seed solution pH (4.5, 5.0, 5.5), rice amount of fermentation bottle (20 g, 30 g, 40 g) and fermentation time (14 d, 17 d, 20 d). The results show that (Table 1), the primary and secondary factors affecting MK yield are in the order of: B>A>D>C, *i.e*., initial pH > temperature > fermentation time > rice amount. Further analysis of variance and significance test showed that factors A (temperature) and B (pH) had extremely significant effects on MK yield (p < 0.01). By comparing the magnitude of K, the optimal levels of the four factors were determined as A2, B2, C3 and D2, that is, pre-fermentation temperature 30°C, seed solution pH 5.0, rice amount in the fermentation bottle 40 g and fermentation time 17 d. The MK yield under the fermentation conditions was 4.64 mg/g (Note: the inoculum size was 10%, the number of air holes was adjusted to 5 wells, no additional carbon source, nitrogen source and sodium acetate were added), which was 28.5% higher than before optimization, and about 8.3 times that of commercially available Gutian red yeast rice.

Study on Cholesterol-lowering Effect of High MK Red Yeast Rice

The results show that (Fig. 13–14): before administration, the TC values of the serum of B, C, D, E groups are 42.6% higher than that of A group (P<0.05); the TG value is about 38.5% higher than that of A group (P <0.01). This indicates that the experimental hyperlipidemia model of KM mice is successfully

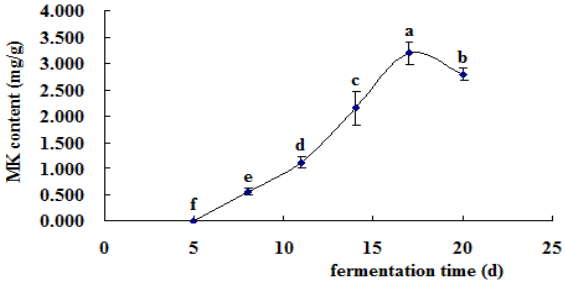

Fig. 8: Effects of fermentation time on the production of MK

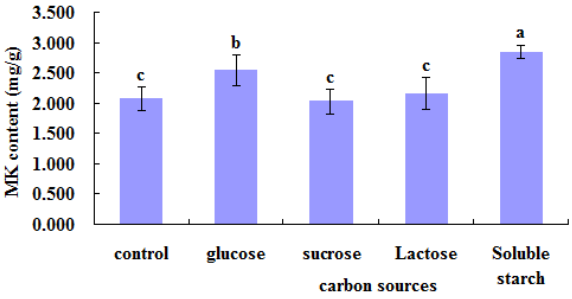

Fig. 9: Effects of additional carbon sources on the production of MK

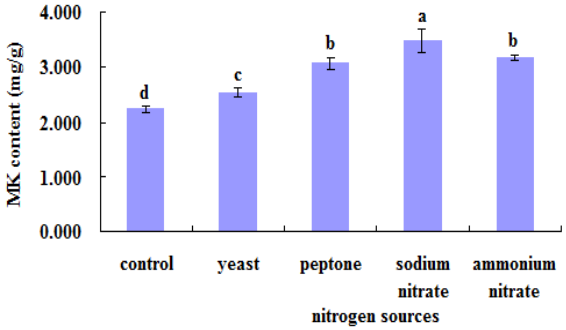

Fig. 10: Effects of additional nitrogen sources on the production of MK

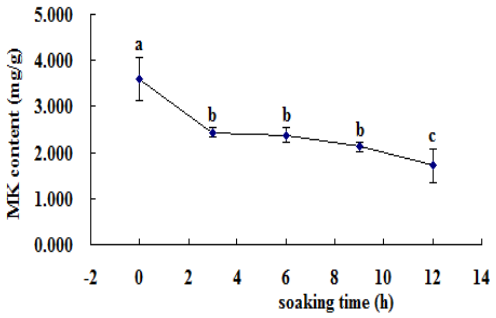

Fig. 11: Effects of rice soaking time on the production of MK

Table 1: The results and analysis of L_9 (3^4) orthogonal test

Test number	factors				MK yield (mg/g)
	A (temperature of fermentation prophase / °C)	B (pH of seed liquid)	C (weigh of rice / g)	D (fermentation time / d)	
1	28	4.5	20	14	3.062
2	28	5.0	30	17	4.375
3	28	5.5	40	20	3.584
4	30	4.5	30	20	3.719
5	30	5.0	40	14	4.510
6	30	5.5	20	17	3.825
7	32	4.5	40	17	3.277
8	32	5.0	20	20	3.616
9	32	5.5	30	14	3.028
K1	3.674	3.353	3.501	3.533	
K2	4.018	4.167	3.707	3.826	
K3	3.307	3.479	3.790	3.640	
R	0.711	0.814	0.289	0.293	

Table S2: Effects of 26 pcs. rice fermented by strain FG-2 on the production of MK

Rice raw material	MK yield (mg/g)	Significance level of difference (P< 0.05)	Significance level of difference (P< 0.01)
1a825	3.61±0.31	a	A
e201	2.28±0.20	b	B
1a820	1.87±0.16	c	C
Dulimi (CK1)	1.33±0.12	d	D
1a1476	1.26±0.11	de	DE
1A825	1.23±0.11	de	DE
1a1461	1.17±0.10	def	DE
1A1461	1.02±0.09	ef	DEF
1a817	0.92±0.08	fg	EF
1a819	0.70±0.06	gh	FG
Gutian red yeast rice (CK2)	0.56±0.06	h	G
1a1463	0.55±0.05	h	G
1a1473	0.54±0.05	h	G
1A819	0.52±0.05	h	G
1A817	0.51±0.05	h	G
1A1463	0.51±0.05	h	G
E201	0.50±0.04	h	G
1A820	0.50±0.05	h	G
1A1473	0.50±0.04	h	G
1A1476	0.49±0.03	h	G
1a816	0.49±0.04	h	G
AXN	undetected	i	H
1A814	undetected	i	H
1a814	undetected	i	H
n84	undetected	i	H
axn	undetected	i	H
1A816	undetected	i	H
N84	undetected	i	H

For the number of rice raw materials, lower case indicates milled rice and upper case indicates brown rice.

modeled. After three weeks of administration, the TC values of C, D and E groups were 29.5%, 34.1% and 37.0% lower than those of group B (P<0.01); The TG values were 3.7, 4.9 and 7.9% lower than those of group B, respectively, without significant difference. This shows that high MK red yeast rice prepared herein and the commercially available Gutian red yeast rice can significantly reduce the total cholesterol level in mice.

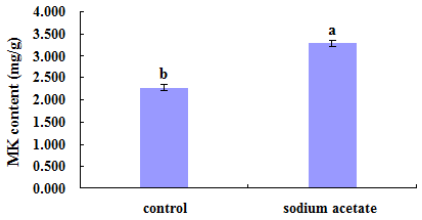

Fig. 12: Effects of additional precursors on the production of MK

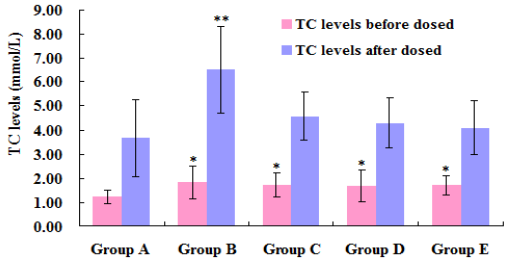

Fig. 13: TC levels in the serum of mice in each test group before administration and three weeks after administration. * indicates significant difference between B, C, D, E groups and A group before the administration (P < 0.05); ** indicates extremely significant difference between B group and A group after administration (P < 0.01)

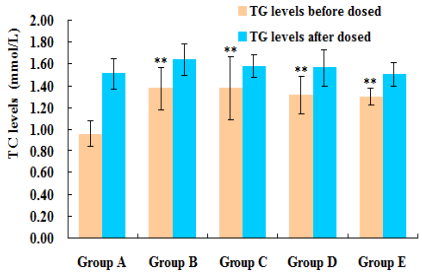

Fig. 14: TG levels in the serum of mice in each test group before administration and three weeks after administration. ** indicates extremely significant difference between B, C, D, E groups and A group before the administration (P < 0.01)

Discussion

Studies had shown that different substrates, such as rice, millet, wheat and barley, sorghum, cassava, sweet potato and potato, have an important influence on MK yield of *Monascus* fermentation (Lee *et al*., 2006; Subhagar *et al*., 2009; Venkateswaran and Vijayalakshmi, 2010; Zhang *et al*., 2018). Priatni *et al*. (2014) used tofu, rice and *Dioscorea hispida* respectively as the substrate to carry out solid fermentation of *Monascus*. The results showed that MK yield was the highest in rice 4.6 times of *Dioscorea hispida* and 4.3 times of tofu. Panda *et al*. (2010) found that the predicted MK yield was 2.83 mg/g and the actual MK yield was 2.80 mg/g when rice used as the substrate, indicating that the rice-based fermentation efficiency was as high as 98.93%. Therefore, rice may be an excellent substrate for the fermentation of *Monascus*.

Pengnoi *et al*. (2017) used different varieties of rice as the matrix for *Monascus* fermentation. The results showed that MK yield (13.48 mg/kg) of rice variety Doi Muser as a matrix was significantly higher than other varieties of rice. This indicated that different rice varieties also had a significant effect on MK yield in *Monascus* fermentation. In addition, the viscosity of rice has a great influence on MK yield. Because of its relatively high viscosity and easy agglomeration, glutinous rice is not conducive to *Monascus* growth (Pattanagul *et al*., 2007). This study also showed that MK yield of the fermentation product is extremely low, even without detection when the rice (all glutinous rice) numbered "1a816", "1a814", "n84", "axn", "1A814", "1A816", "N84", "AXN" are used as the raw material. This suggests that the glutinous rice is not suitable as a substrate for *Monascus* fermentation (Table S2) shown). It is possible that glutinous rice is bound into clusters after being cooked, the poor gas permeability hinders the mycelial growth of *Monascus*, thereby further reducing MK yield and even producing no MK. In addition, MK yield of polished rice as raw material is higher than brown rice in the same variety (Table S2). It is possible that the brown rice has a skin layer and an aleurone layer on the surface, making *Monascus* mycelium unable to fully utilize the rice nutrients, and consequently leading to lower MK yield. In summary, selection of polished rice of non-glutinous rice varieties as a raw material for solid fermentation of *Monascus* can help increase MK yield of red yeast rice.

The solid-state fermentation condition for lovastatin produced by *A. terreus* is similar to MK produced by *Monascus* (Kumar *et al*., 2000). The carbon nitrogen ratio of the matrix is also one main factor affecting the yield of lovastatin by *A. terreus*. The yeast extract and soybean meal are the preferred nitrogen sources. The carbon in the lovastatin synthesis pathway is much lower than the carbon converted to biomass. Therefore, nitrogen limitation (*i.e*., growth inhibition) helps divert more carbon to the synthesis of lovastatin (Lopez *et al*., 2003).

Miranda *et al*. (2014) showed that reactive oxygen species (ROS) would be produced during the fermentation of *A. terreus* and the ROS concentration was positively correlated with the lovastatin yield. ROS could increase the expression of *Lov E* and *Lov F* in the lovastatin biosynthetic gene cluster, thereby increasing lovastatin yield. When antioxidants were added, significant reduction was observed in expression levels of lovastatin biosynthetic gene clusters and lovastatin yield. This indicates that oxidative stress can promote the synthesis of lovastatin or MK, and reactive oxygen species can significantly increase the content of lovastatin or MK in the fermentation product.

Conclusion

In this study, the optimal combination of MK high-yield strains and rice raw materials was screened out: *Monascus purpureus* strain FG-2 and polished rice of rice variety "1a825" and MK yield was 3.2 times of the control strain and 2.7 times of the control rice raw material, respectively. The optimum fermentation conditions for the combination were: pre-fermentation temperature 30°C, seed solution pH 5.0, rice amount in fermentation bottle 40 g, fermentation time 17 d; the MK yield was 4.64 mg/g under the fermentation condition, which was 28.5% higher than before fermentation condition optimization, and about 8.3 times of commercially available Gutian red yeast rice. The high MK red yeast rice prepared herein has better cholesterol lowering effect than the commercially available Gutian red yeast rice.

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