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Red Yeast Rice Fermentation by Selected *Monascus* sp. with Deep-Red Color, Lovastatin Production but No Citrinin, and Effect of Temperature-Shift Cultivation on Lovastatin Production

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Abstract *Monascus pilosus* NBRC4520 was selected for functional fermented food inoculation for its high lovastatin and low citrinin production with a deep-red color. For koji (mold rice) with high lovastatin production, separation of the growth phase and lovastatin production phase by shifting the temperature from 30 to 23 °C increased lovastatin production by nearly 20 times compared to temperature-constant cultivation. In addition, citrinin was not produced even in the lovastatin production phase, although the pigment was increased. With temperature-shift cultivation, 225 μg lovastatin/g dry koji was produced in 14 days without citrinin.

Keywords Monascus · Lovastatin · Temperature-shift cultivation · Pigment · Citrinin

Introduction

A fungal species, *Monascus*, has been used for traditional fermented food and has attracted attention because of its biologically active metabolite production, such as monacolin K (lovastatin), and γ-amino butyric acid (GABA) [1]. Monacolin K is a secondary metabolite of *Monascus* strains, and Endo [2] discovered that *M. ruber* produces an active methylated form of compactin known as monacolin K (lovastatin; mevinolin) in liquid fermentation. Monacolin K functions as an inhibitor of 3-hydroxy-3-methyl glutaryl-coenzyme A reductase, which is a regulatory and rate-limiting enzyme of cholesterol biosynthesis [2].

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GABA has several physiological functions, such as neurotransmitting, hypotensive, and diuretic effects, which were reported to be affected by culture conditions either in submerged or solid cultures [3].

Monascus-derived fermented foods have been also characterized by its deep-red color; therefore, they are called red-yeast rice or red-mold rice. *Monascus* strains have long been used for the production of pigments that have been typically used, particularly in East Asia, as a coloring agent for foodstuffs [4].

In contrast to the advantage of *Monascus* as a food microorganism, some strains of *Monascus* were reported to produce citrinin, a nephrotoxin, which was previously found in *Aspergillus* and *Penicillium* genera and might contaminate in *Monascus*-derived food. Repression of citrinin production in *Monascus* has been reported using genetic disruption [5], culture condition optimization, and mutation [6].

Based on the background of *Monascus* sp. as a functional food fermentant, fungi expressing Monacolin and/or GABA with a deep-red color, but no citrinin, would be desirable; however, the correlation between the pigment and citrinin production is not clear.

In this study, we screened for a suitable *Monascus* fungus for fermented food with high lovastatin and low citrinin production with a deep-red color, followed by an examination of red koji using two-step temperature-shift cultivation.

Materials and Methods

Fungal Strains and Cultivation

Twenty-nine strains of *Monascus* spp. were used in this study, as listed in Table 1. To maintain the culture, strains were grown on a potato dextrose agar (PDA, Difco, CA, USA) slant followed by storage at 4 °C. For koji (rice mold) preparation, ca. 50 g (for Table 2), or

Table 1 *Monascus* strains used in this study.

Strains	Reference no.	Strains	Reference no.
Monascus purpureus	NBRC 4478[a]	*Monascus ruber*	NBRC 9203
Monascus purpureus	NBRC 4485	*Monascus purpureus*	NBRC 30873
Monascus purpureus	NBRC 4482	*Monascus purpureus*	NBRC 32316
Monascus pilosus	NBRC 4521	*Monascus ruber*	NBRC 32318
Monascus pilosus	NBRC 8201	*Monascus purpureus*	NBRC 32228
Monascus ruber	NBRC 4483	*Monascus purpureus*	ATCC 26264
Monascus ruber	NBRC 4532	*Monascus purpureus*	ATCC 48162
Monascus pilosus	NBRC 4480	*Monascus kaoliang*	ATCC 46595
Monascus purpureus	NBRC 4484	*Monascus kaoliang*	ATCC 46596
Monascus purpureus	NBRC 4486	*Monascus kaoliang*	ATCC 46597
Monascus pilosus	NBRC 4487	*Monascus kaoliang*	ATCC 46598
Monascus purpureus	NBRC 4489	*Monascus* sp.	ATCC 16434
Monascus pilosus	NBRC 4520	*Monascus* sp.	ATCC 16437
Monascus purpureus	NBRC 6085	*Monascus* sp.	ATCC 34570
Monascus purpureus	NBRC 6540		

[a] Formerly registered as IFO

Table 2 Summary of citrinin, lovastatin, and pigment production by *Monascus* spp.

Strains		Citrinin (μg/g koji)	CTpks[a] (%)	Lovastatin (μg/g koji)	Color[b]
High citrinin producer					
M. purpureus	NBRC 4478	94,387	+ (100)	nt	0.04
M. purpureus	NBRC 4482	184,351	+ (100)	nt	1.15
M. purpureus	NBRC 4486	12,554	+ (100)	nt	1.54
M. purpureus	NBRC 6540	145,445	+ (100)	nt	2.51
M. purpureus	NBRC 30873	29,287	+ (100)	nt	2.81
M. purpureus	NBRC 32316	16,840	+ (100)	nt	2.98
M. purpureus	NBRC 32228	39,391	+ (100)	nt	3.96
M. purpureus	ATCC 26264	15,924	+ (100)	nt	3.98
M. purpureus	ATCC 48162	19,631	+ (100)	nt	4.43
M. kaoliang	ATCC 46595	2,575	+ (100)	nt	6.01
M. kaoliang	ATCC 46596	38,185	+ (100)	nt	6.93
M. kaoliang	ATCC 46597	44,423	+ (100)	nt	7.27
M. kaoliang	ATCC 46598	2,809	+ (100)	nt	7.62
Monascus sp.	ATCC 16437	48,063	+ (100)	nt	8.32
Monascus sp.	ATCC 34570	12,213	+ (100)	nt	8.39
Low citrinin producer					
M. purpureus	NBRC 4485	346	+ (100)	20.8	0.73
M. pilosus	NBRC 4521	82	–	8.3	1.20
M. pilosus	NBRC 8201	101	–	75.0	1.30
M. ruber	NBRC 4483	108	–	15.0	1.40
M. ruber	NBRC 4532	75	–	50.0	1.46
M. pilosus	NBRC 4480	104	–	314.8	1.48
M. purpureus	NBRC 4484	398	–	0	1.52
M. pilosus	NBRC 4487	100	–	0	1.57
M. purpureus	NBRC 4489	161	+ (100)	0	1.59
M. pilosus	NBRC 4520	165	–	565.3	2.17
M. purpureus	NBRC 6085	721	–	nt	2.39
M. ruber	NBRC 9203	59	–	202.5	2.56
M. ruber	NBRC 32318	36	–	405.5	3.31
M. ruber	ATCC 20657	50	–	57.0	5.03
Monascus sp.	ATCC 16434	65	+ (97.9)	0	7.77

Strain used for temperature-shift cultivation is underlined

nt not tested

[a] Nucleotide identity with CTpks (accession no. AB243687)

[b] Expressed as 100*OD at 500 nm

1 kg (Fig. 2), of moistened rice in 200-ml Erlenmeyer flasks were steamed with autoclave at 121 °C for 5 min, followed by the inoculation of 1 ml conidial suspension (5×10^5-conidia/ml) of *Monascus* spp. were inoculated to the rice with 4 ml of sterilized deionized water. They were then incubated at 30 °C for 4 days, followed by further incubation at 25 °C for additional 17 days (Table 2) or 10 days (Fig. 2), with daily mixing aseptically. YPD medium (Difco, CA, USA) was prepared for genomic DNA preparation. Unless otherwise stated, cultivations were carried out at 30 °C.

Genetic Manipulations

Genomic DNA from *Monascus* spp. were extracted from YPD medium-grown mycelium with ISOPLANT II (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. The PKS homologue gene was amplified by polymerase chain reaction (PCR) with a primer set designed from the CTpks gene of *M. purpureus* (Genbank accession no. AB243687), CTpksf GACACGGCATGCTCGTCAT and CTpksr ACTCACCAAA GCTGTGCCC under the following conditions: 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min, and a final elongation step at 72 °C for 10 min.

Sequencing PKS homolog genes amplified by PCR were subcloned in pT7-blue vector (Takara-bio, Shiga, Japan) followed by sequencing on both strands by the vector primer with the ABI model 3100 and Big Dye terminator kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions.

Analysis Cellular growth at various temperatures was examined by measuring the colony diameter grown on PDA plate. For citrinin and pigment analysis, 20–50 g of the koji was sampled, followed by the crushing with Oster Blender ST-1 (OSAKA CHEMICAL Co., Ltd., Osaka, Japan) for 2 min, extraction with 2.5 volume of 70% ethanol with extensive voltexing for 1 h. Total pigment production was determined as described by Tseng et al. [7]. Citrinin was analyzed using a RIDA screen FAST citrinin kit (Adumax Co., Tokyo, Japan) based on ELISA. Lovastatin was measured by HPLC using a Shimadzu 10A (Shimadzu Co., Kyoto, Japan) under the following conditions: column: Wakosil-II 5C18 with 4.6 mm ID and 250 mm length (Wako Pure Chemicals Co., Tokyo, Japan); oven temperature, 40 °C; flow rate, 0.5 ml/min, after isocratic elution with methanol—0.1% phosphate (72:28, *v*/*v*) for 25 min followed by linear gradient from methanol—0.1% phosphate (72:28, *v*/*v*) to methanol—0.1% phosphate (100:0, *v*/*v*) until 30 min; UV detection at 238 nm. All the data were determined by at least duplicate analysis.

Results and Discussion

Selection of Fungi Producing High Lovastatin and High Pigments but No Citrinin

In order to select *Monascus* spp. suitable for added-value fermentation seeds, we selected using three indexes, lovastatin, pigment, and citrinin productions. Figure 1 shows the profiles of the examined strains with regard to pigmentation and citrinin production in solid culture. There was no clear correlation between the pigment (absorbance at 500 nm) and citrin production. Table 2 summarizes the results of the selection using citrinin, pigment, and lovastatin production for the strains used in this study. Of the 31 strains tested, 16 strains exhibited citrinin production less than 1,000 μg/g koji under the conditions in this study and subjected to lovastatin production. Ten of the 16 produced lovastatin and four strains produced higher than 100 μg/g koji.

The gene responsible for citrinin production, PKScit, was further amplified to identify the correlation between citrinin phenotypic production and the detected genes. PKScit was amplified from genomic DNA of *M. purpureus* and *M. kaoliang* with identity higher than 97%. Since there has been only one report on PKScit from *M. purpureus* [5], other genes could have high similarity in *M. kaoliang* while low similarity in *M. ruber* and *M. pilosus.*

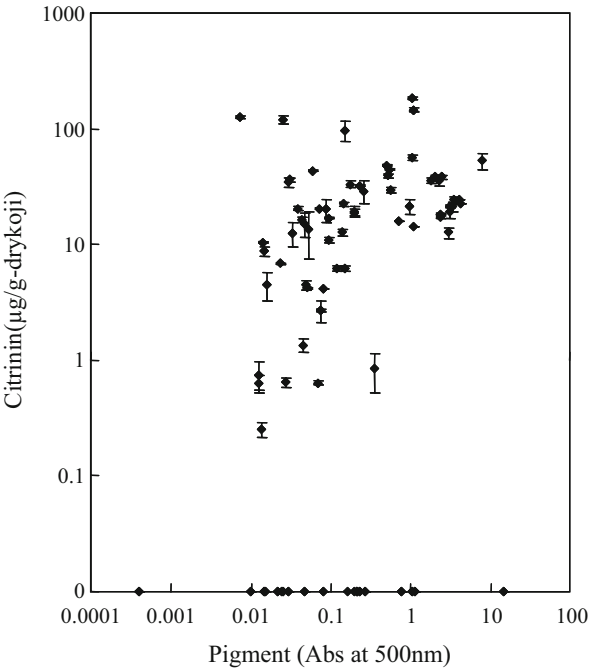

Fig. 1 Relationships between citrinin production and pigment production among various *Monascus* spp. *Error bars* represent the standard deviation from the mean value of duplicate experiments

It is also reported that *M. ruber* and *M. pilosus* are evolutionarily closer than *M. purpureus* by phylogenetic analysis using β-tublin sequences [8].

Based on the results described above, as strain *M. pilosus* NBRC4520 showed the highest lovastatin production with comparable pigment production, we selected strain *M. pilosus* NBRC4520 for further study.

Production of Lovastatin by Temperature Shift Solid Fermentation

Separation of cellular growth and production phase could contribute to significantly increase productivity. Preliminary growth test showed that the strain NBRC4520 grew from 20 to 42 °C, and the optimal temperature was found to be 30 °C (data not shown). After solid culture was carried out at 30 °C for 4 days, each culture was transferred to various conditions, as shown in Table 3. Although anaerobic conditions produced no lovastatin, cultivation at 23 °C under aerobic conditions increased production after 6 days, compared to at 30 °C, the optimal temperature for cellular growth. It was previously reported that lovastatin, citrinin, and GABA productions were affected by various culture conditions including temperature. In *M. purpureus* NTU601, the highest lovastatin production was obtained at 30 °C, while no examination with temperature-shift cultivation was carried out [9]. It is also noted that lower temperature (25 °C) gave less lovastatin productivity in the strain NTU601, while higher production at 23 °C than that at 30 °C in case of our study.

Figure 2 shows the detailed time course of temperature-shift cultivation under aerobic conditions in koji (rice mold) preparation in a large scale. After shifting the temperature from 30 to 23 °C on the 4th day, lovastatin started to be produced linearly until the 12th day with pigment production. On the 14th day, lovastatin production reached 225 μg/g dry koji

Table 3 Lovastatin produced by strain NBRC 4520 at various temperatures.

Temp. (°C)[a]		Cultivation days	
		6	17
30	Aerobic	18.7	8.9
	Anaerobic	0	0
23	Aerobic	53.2	157.2
	Anaerobic	0	9

[a] Temperature was changed to the stated value after cultivation at 30 °C for 4 days

Values are expressed as μg/g dry koji

without producing citrinin. No citrinin production in this case may be due to the shorter cultivation time than that for Table 2 or caused by scaling up from 50 g to 1 kg. This should be further examined using the 1 kg production scale. In contrast, no lovastatin was detected also in this scale when the temperature was constant at 30 °C, as expected from data in Table 3 (data not shown). Regarding the effect of physical culture conditions for *Mosascus* spp., the effect of light irradiation was reported [10]. Their study shows that red light enhanced GABA, red pigments, monacolin K, and citrinin, in a submerged culture of *M. pilosus* and *M. purpureus*. In a solid culture, nutrients enhancing secondary metabolites of *Monascus* spp. have been extensively investigated, such as sodium nitrate [3], water and ethanol [10]. In the large scale production, lovastatin production nearly stopped at 225 μg/g dry koji while higher productivity was obtained in a small scale (565 μg/g dry koji in Table 2). Examinations for further increased production are currently in progress either by nutrients feeding or operational conditions optimization.

Conclusion

Temperature-shift cultivation has been applied as a simple technology to improve production in submerged culture [11]. Here, we report the possibility of temperature-shift

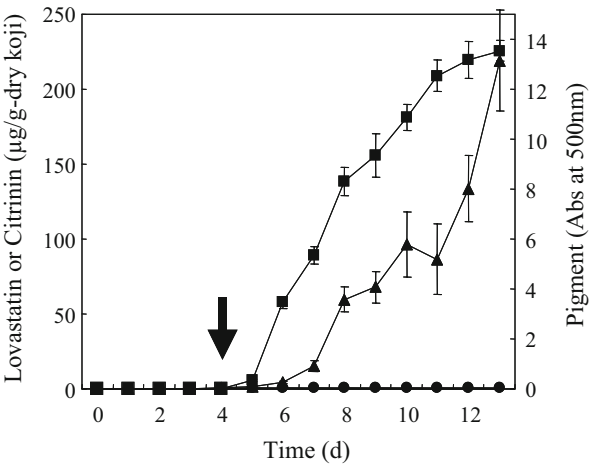

Fig. 2 Time course of citrinin, lovastatin, and pigment production by *M. pilosus* NBRC 4520 during koji (rice mold) cultivation. *Circles*: citrinin (μg/g dry koji), *triangles*: pigment (Abs at 500 nm), and *squares*: lovastatin (μg/g dry koji). Temperature was changed from 30 to 23 °C at 4 days (*arrow*). *Error bars* represent the standard deviation from the mean value of triplicate experiments

cultivation for lovastatin production by *Monascus* spp. Initial cultivation at optimal growth temperature followed by preferential lovastatin production at lower temperature, suppressing cellular growth, could also be applied for other *Monascus* spp. exhibiting higher lovastatin productivity. We are currently examining the details of condition optimization, such as nutrient supply, to improve the production of both lovastatin and pigment.

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References

1. Lin, Y. L., Wang, T. H., Lee, M. H., & Su, N. W. (2008). Biologically active components and nutraceuticals in the *Monascus*-fermented rice: a review. *Applied Microbiology and Biotechnology*, *77*, 965–973. doi:10.1007/s00253-007-1256-6.
2. Endo, A. (1979). Monacolin K, a new hypocholesterolemic agent produced by a *Monascus* species. *The Journal of Antibiotics*, *32*, 852–854.
3. Su, Y. C., Wang, J. J., Lin, T. T., & Pan, T. M. (2003). Production of the secondary metabolites gamma-aminobutyric acid and monacolin K by *Monascus*. *Journal of Industrial Microbiology & Biotechnology*, *30*, 41–46.
4. Babitha, S., Soccol, C. R., & Pandey, A. (2007). Solid-state fermentation for the production of *Monascus* pigments from jackfruit seed. *Bioresource Technology*, *98*, 1554–1560. doi:10.1016/j.biortech.2006.06.005.
5. Shimizu, T., Kinoshita, H., Ishihara, S., Sakai, K., Nagai, S., & Nihira, T. (2005). Polyketide synthase gene responsible for citrinin biosynthesis in *Monascus* purpureus. *Applied and Environmental Microbiology*, *71*, 3453–3457. doi:10.1128/AEM.71.7.3453-3457.2005.
6. Suh, S. H., Rheem, S., Mah, J. H., Lee, W., Byun, M. W., & Hwang, H. J. (2007). Optimization of production of monacolin K from gamma-irradiated Monascus mutant by use of response surface methodology. *Journal of Medicinal Food*, *10*, 408–415. doi:10.1089/jmf.2006.097.
7. Tseng, Y. Y., Chen, M. T., & Lin, C. F. (2000). Growth, pigment production and protease activity of *Monascus purpureus* as affected by salt, sodium nitrite, polyphosphate and various sugars. *Journal of Applied Microbiology*, *88*, 31–37. doi:10.1046/j.1365-2672.2000.00821.x.
8. Park, H. G., Stamenova, E. K., & Jong, S. - C. (2004). Phylogenetic relationships of *Monascus* species inferred from the ITS and the partial β-tubulin gene. *Botanical Bulletin of Academia Sinica*, *45*, 325–330.
9. Wang, J. J., Lee, C. L., & Pan, T. M. (2003). Improvement of monacolin K, gamma-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601. *Journal of Industrial Microbiology & Biotechnology*, *30*, 669–676. doi:10.1007/s10295-003-0097-2.
10. Miyake, T., Mori, A., Kii, T., Okuno, T., Usui, Y., Sato, F., et al. (2005). Light effects on cell development and secondary metabolism in *Monascus*. *Journal of Industrial Microbiology & Biotechnology*, *32*, 103–108. doi:10.1007/s10295-005-0209-2.
11. Ansorge, M. B., & Kula, M. R. (2000). Investigating expression systems for the stable large-scale production of recombinant L-leucine-dehydrogenase from *Bacillus cereus* in *Escherichia coli*. *Applied Microbiology and Biotechnology*, *53*, 668–673. doi:10.1007/s002539900290.