Kasetsart J. (Nat. Sci.) 43 : 696 - 702 (2009)

Pigments and Anti-Cholesterol Agent Production by *Monascus kaoliang* KB9 and Its Color Mutants in Rice Solid Cultures

Jirawun Chayawat[1], Saeree Jareonkitmongkol[1], Apisit Songsasen[2] and Busaba Yongsmith[1]*

ABSTRACT

A comparative study of four *Monascus* strains, (a red-pigment-producing *Monascus kaoliang* KB9 strain and its three color mutants of red, yellow and white) on various agar media revealed different colony and pigment characteristics. Three agar media: MYS, GYP and PDA gave clear cultural characteristics. Upon varying medium formulas, the wild type and the red mutant showed red pigmentation, while the yellow and white mutants showed deep orange and white pigmentation, respectively. All strains were grown on rice solid culture and examined for the production of pigment and anti-cholesterol agent using a spectrophotometer and a modified HPLC method, respectively. The results showed that the wild-type strain (KB9) and its red mutant (KB10M16) could produce substantial amounts of anti-cholesterol agent compared with the yellow and white mutants (KB20M10.2 and KB20M1). Due to its good growth and production of the greatest amount of anti-cholesterol agent, the wild-type KB9 strain was selected for a study on the optimization of anti-cholesterol agent fermentation. Under favorable conditions of 64% relative humidity at room temperature (28-30°C) for 5 weeks incubation, production of the anti-cholesterol agent and red pigment was high, with 17,892 mg/kg and 4,640 Unit (OD500) and 4,834 Unit (OD400) per gram dried weight, respectively.

Key words: *Monascus* spp., color mutants, pigments, anti-cholesterol agent**,** rice solid culture

INTRODUCTION

Monascus spp. are well known for their ability to produce many bioactive, secondary metabolites including food pigments and a traditional medicine, monacolin K, in China and Asian countries (Ma *et al*., 2000). The color of the fungus varies from yellow to orange, red and purple-red, depending on culture conditions (Wong *et al*., 1981). The anti-cholesterol agent, monacolin K, was discovered in the broths of *Monascus ruber* by Endo (1979). The monacolin K (lovastatin or mevinolin) content in fermented substrates is another functional value-added application of the *Monascus* mold, which inhibits cholesterol formation by stopping the action of a key enzyme in the liver, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol biosynthesis (Manzoni and Rollini, 2002). The optimal cultivation temperature for

1 Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author: e-mail: fscibus@ku.ac.th

Received date : 15/05/08 Accepted date : 16/07/09

monacolin production is between 24 to 26°C, but is higher for pigment production from the same strain (Gailing, 1998).

In the authors' laboratory, several strains of *Monascus* spp. have been accumulated, which have the capability to produce different pigments using the same cultivation conditions. The wild-type strain is *Monascus kaoliang* KB9, an excellent cassava starch-utilizing red fungus. Improvement of this wild type by UV-irradiation for 10 min results in the red mutant, *Monascus* sp. KB10M16, which produces a higher yield of red pigment. A UV exposure time of 20 min sequentially applied to the red mutant, gave a new yellow mutant, *Monascus* sp. KB20M10.2, which produced yellow pigment (λmax at 370 nm) instead of the red pigments (μmax at 420 and 500 nm) produced by its parents (Yongsmith *et al*., 2000). In addition, one white mutant or albino mutant, *Monascus* sp. KB20M1, was derived from the wild type by UV-irradiation for 20 minutes. Jongrungruangchok *et al*. (2004) found a crude CH_2Cl_2 extract of *M. kaoliang* KB20M10.2 grown on rice that was sequentially subjected to Sephadex LH-20 and Si gel chromatography, to yield two new azaphilone yellow pigments, monascusone A and monascusone B, together with two known compounds, monascin and FK17-P2b2. However, either crude ethanol yellow extract or its key purified yellow pigment (monascusone A) could serve as a strong antioxidant analyzed by FRAP or DPPH assay (Harntrikanon, 2006).

In addition, the various colorants from the *Monascus* molds produced in this study could inhibit the formation of mutagens in the reaction of sodium nitrite with 1-aminopyrene or fish extract using Ames test (Kruawan *et al*., 2005). Dontri *et al*. (2008) first reported the dual effects of red mold rice (RMR) of *Monascus kaoliang* KB9 supplementation at 0.5-2.50% in the diets of laying hens compared with the negative and positive controls over a three-month period. They reported that with RMR present at a level of at least 0.5% (w/w), there was a reduction of 30.1 and 23.2% in serum cholesterol and egg cholesterol, respectively, and an increase of 32.49% in egg yolk pigment compared with the negative control. The present paper aimed to compare the production of pigments and their anti-cholesterol agent in the wild type and its three color mutants in order to select the potential strain for broadening its application.

MATERIALS AND MEDTHODS

Microorganisms and cultivation

The wild type (*Monascus kaoliang* KB9) a red mutant (KB10M16), a yellow mutant (KB20M10.2) and a white mutant (KB20M1) were maintained in MYS agar (Yongsmith *et al*., 1990; Yongsmith *et al*., 1994). The solid-culture media used for the study on the culture characteristics were MYS, GYP, and Potato Dextrose Agar (PDA). The GYP medium was composed of 4% glucose, 1% yeast extract and 0.5% peptone.

Rice solid fermentation of *Monascus* strains

Monascus strains were cultured at room temperature (28-30°C) for 7 days on C medium (Hiroi *et al*., 1979). Conidia that formed were collected and suspended in sterile 0.1% (v/v) Tween 80. Two milliliters of 10^6 spores/ml of *Monascus* molds were inoculated on a local cultivar of non-sticky rice, known as the Kao-Hom Mali cultivar. All strains were incubated on moistened rice in Erlenmeyer flasks for one or five weeks in an incubator at 64% relative humidity with different temperature conditions: 28-30°C (RT); 3 d at 28-30°C and then at 26°C (RT26); and 26°C (26C). The preparation of the rice medium for solid culture involved the following procedure. Dehulled rice was soaked in tap water for 2 h. After the water was removed, the soaked rice was drained for 10 min and then a 500-ml flask containing 100 g rice was autoclaved for 15 min at 121.5°C and cooled to room temperature (Yongsmith *et al*., 2000).

Estimation of pigment concentration

The pigment concentration was measured using a Shimadzu spectrophotometer model UV-1700 at 370, 400, 420 and 500 nm. Pigment in fermented rice samples corresponding to 1 g of initial rice substrate was extracted with 39 ml of 70% ethanol for 9 h on a rotary shaker (300 rpm). The extract was then filtered to remove suspended solids and the supernatant was analyzed by a spectrophotometer against a 70% ethanol blank. The moisture content of each rice sample was determined by heating the fermented rice in a hot air oven overnight at 105°C and the weight loss was measured (Yongsmith *et al*., 2000).

Anti-cholesterol agent analysis

After fermentation, the fermented rice was steamed at 100°C for 30 min, dried at 50°C for 24-36 h and then ground to powder of which 0.5 g was extracted with 25 ml of 70% ethanol at 50°C for 2 h, follawed by filtration through a 0.2 μm filter membrane and then measured by an HPLC system.

HPLC condition for anti-cholesterol agent analysis

The HPLC system consisted of a Shimadzu LC-10AT VP Liquid Chromatograph, a FCV-10AL VP pump, an LDC Analytical SpectroMonitor 3100 detector set at 238 nm and an LDC Analytical CI-4100 integrator. Chromatography columns Persuit C18, 5μm, 250×4.6 mm were connected to a guard column (MetaGuard Pursuit 4.6 mm 5μ C18) and 20 μl loop injector. An isocratic mobile phase of acetonitrile:water at the ratio of 65:35 (by vol.) was used. The flow rate was 1.0 ml/min at a temperature of 28°C (Ganrong *et al*., 2003). Monacolin K (Sigma) was dissolved in 70% ethanol to prepare the standard monacolin K.

RESULTS AND DISCUSSION

Cultural characteristics of *Monascus* spp.

Monascus kaoliang KB9 (wild type) and a red mutant, yellow mutant and white mutant were cultured on MYS, GYP and PDA media at room temperature (28-30°C) with 7 d incubation and used to study cultural characteristics. Figure 1

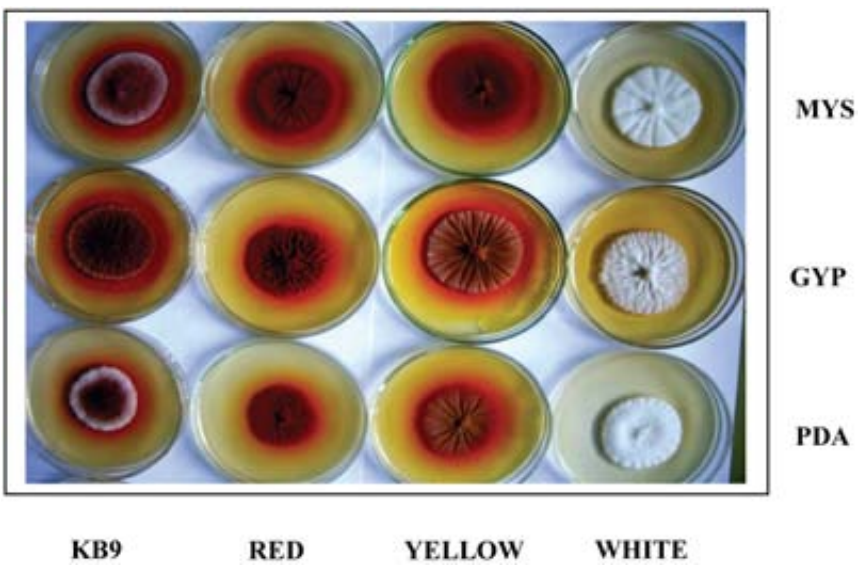

Figure 1 Culture characteristics of *Monascus kaoliang* KB9 and its color mutants on MYS, GYP and PDA after 7 d of incubation at room temperature (28-30°C).

shows that the colony formations amongst the four strains were different in growth and pigment characteristics. The colonies of KB9 and the red mutant showed red pigmentation, while dark orange and white pigmentation were found in the yellow and white mutants, respectively. Even though the colony diameters of the red mutant on various medium formulas were less than those of the wild-type KB9, a deeper red pigmentation was observed. Characteristics of rice fermented by *Monascus kaoliang* KB9 and its color mutants are shown in Figure 2. Rice solid cultures of KB9 and its red mutant showed a deep red pigmentation while the yellow and white mutants showed dark brown and pastel pigmentation, respectively.

Pigment production

Figure 3 shows the fermentation time for pigment production of the four strains on rice solid cultures in an incubator at 64% relative humidity under different temperature conditions: 28-30°C (RT); 3 d at 28-30°C and then at 26°C (RT26); and 26°C (26C). No pigments could be detected in the white mutant. Under the varying incubation temperature conditions, KB9 and the red mutant could produce pigment with μmax at 400 and 500

Figure 2 Characteristics of rice fermented by *Monascus* strains for 5 weeks at room temperature (28-30°C).

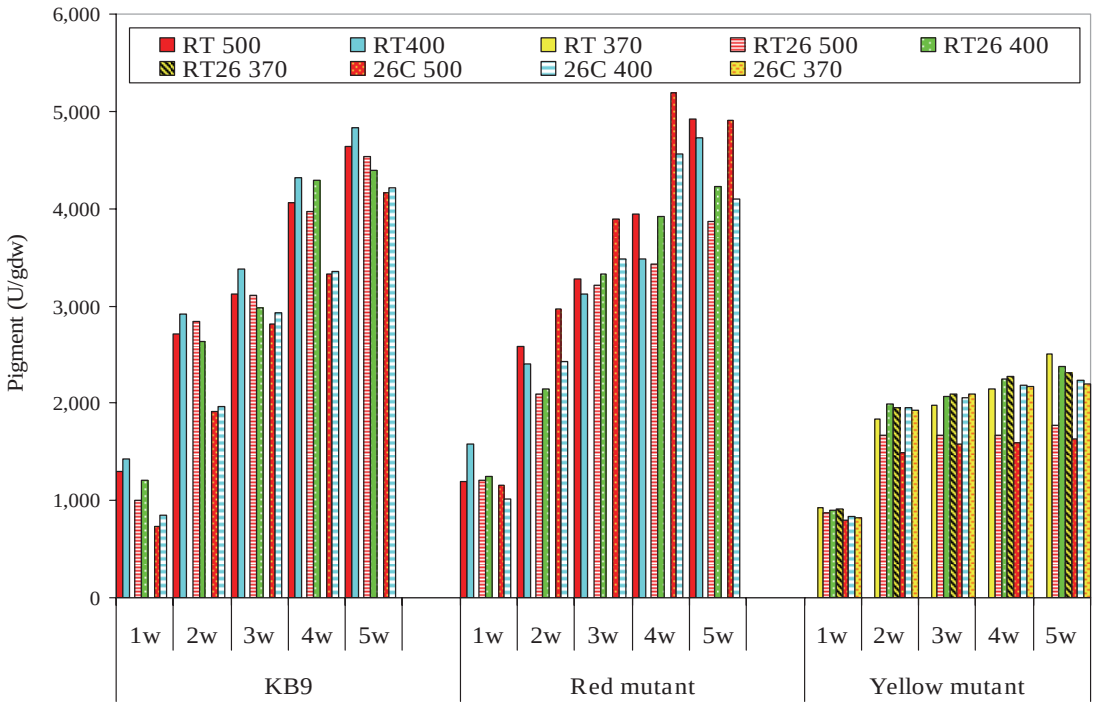

Figure 3 Pigment production of *Monascus* strains in rice solid culture for 1-5 weeks in an incubator at 64% relative humidity at: room temperature of 28-30°C (RT); 3 d at RT and then at 26°C (RT26); and 26°C (26C).

nm only. The KB9 strain reached maximum pigment production within 5 weeks of incubation at room temperature, yielding more pigment at λmax 400 nm than that at λmax 500 nm. The red mutant reached its maximum pigmentation within 4 weeks of incubation at 26°C yielding more pigment at λmax 500 nm than at λmax 400 nm. The yellow mutant could produce yellow pigment with a single μmax of 370 nm at room temperature but could produce yellow pigment with multi λmax of 370, 400 and 500 nm under the RT26 and 26C temperature regimes. Mutagenesis as well as culture temperature could thus affect the various pigment formations of the color mutants.

Anti-cholesterol agent production

The fermentation time for anti-cholesterol agent production of pigment producing strains on rice solid cultures under different temperature conditions are shown in Figure 4. Among the four *Monascus* strains studied, only the red wild-type KB9 and its red mutant were found to produce more anti-cholesterol agent than the yellow mutant did. No anti-cholesteral agent could be detected in the white mutant. Many papers have reported that the optimal temperature for anti-cholesterol agent production is 26°C (Gailing, 1998; Ganrong *et al*., 2003). For example, Ganrong *et al*. (2003) reported that the optimized solid-state fermentation conditions of *Monascus sp*. 9901 are 30°C for *Monascus* growth and 26°C for monacolin K synthesis, resulting in a monacolin K content reaching up to 11,000 mg/kg. However, the red wild-type strain selected in the current study gave a different result. Room temperature (28-30°C) was best for the red pigment and anti-cholesterol agent production, while RT26 was optimal for anti-cholesterol agent production by the red and yellow mutants. No anti-cholesterol agent could be detected in the white mutant. The best production of anti-cholesterol agent in the study occurred with the red wild-type KB9 although it was slow during the first and second weeks of incubation, yielding only 1,701-5,724 mg/kg. After that, the productivity increased to 9,079-17,892 mg/kg in the later phase during the third and fifth weeks of incubation. The anti-cholesterol agent production was related to pigment production in the KB9 strain at week 4 and 5. In this study, the molecular weight of the anti-cholesterol agent from *Monacus kaoliang* KB9 strain analysis by LC-MS was found to be 355 and 359 (data not shown), which differed from a previous report (Li *et al*., 2004). However, the

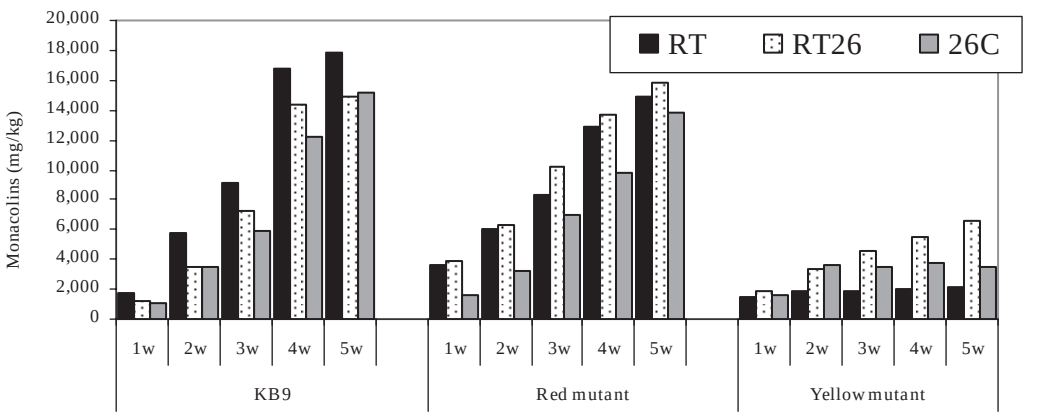

Figure 4 Anti-cholesterol agent fermentation over time in rice solid cultures by *Monascus* strains for 1-5 weeks in an incubator at 64% relative humidity at: room temperature of 28-30°C (RT); 3 d at RT and then at 26°C (RT26); and 26°C (26C).

previous study of Dontri *et al*. (2008) showed that the red mold rice of *Monascus kaoliang* KB9 played a dual function by significantly decreasing the cholesterol of egg yolk and serum, while increasing yolk pigment.

This finding suggested that the potential anti-cholesterol agent might be a new monacolin derivative, which was different from those described in the other reports. Therefore, future study will be necessary for NMR analysis of the chemical structure of the anti-cholesterol agent from red wild-type KB9. In addition, the optimum conditions for anti-cholesterol agent production should be further studied using solid-state fermentation of various substrates with shorter fermentation times and increased mass production for industrial purposes.

CONCLUSION

Among the four strains of *Monascus*, the red-pigment-producing strains (KB9 and its red mutant) were found to produce more anti-cholesterol agent than the yellow mutant did, while none was found in the white mutant. Due to its good growth and the higher production of anti-cholesterol agent, the wild-type KB9 strain was selected for the optimization of anti-cholesterol agent fermentation. Under potentially optimal conditions of 64% relative humidity and room temperature (28-30°C) for incubation over a few weeks, anti-cholesterol agent and red pigment were produced at the high amounts of 17,892 mg/kg and 4,640 Unit (OD500) and 4,834 Unit (OD400) per gram dried weight, respectively. This indicated the possibility of lowering processing costs by using this remarkable strain.

ACKNOWLEDGEMENTS

The authors would like to thank the Kasetsart University Research and Development Institute (KURDI), Kasetsart University, Bangkok, Thailand for financial support.

LITERATURE CITED

Dontri, T, S. Isariyodom, B. Yongsmith, S. Tongyai and N. Sinbuathong. 2008. Supplementation of Red Mold Rice from an Egg Yolk Pigmented and Cholesterol Lowering *Monascus* Strain on Egg Performance and Quality in Laying Hens, pp. 22-26. *In* **The 13th Animal Science Congress of the Asian-Australasian Association of Animal Production Societies Theme: Animal Agriculture and the Role of Small Holder Farmers in a Global Economy Hanoi-Vietnam**.

Endo, A. 1979. Monacolin K, a new hypocholesterolemic agent. **J. Antibiot.** 32: 852-854.

Gailing, L. 1998. The manufacturing of Wenstardin capsule, a new registered and marketable drug based on red rice as raw material. **Meetings/Symposia From 8th to 10th July 1998 in Toulouse, France "The Symposium on *Monascus* Culture and Application"**. [Online] Available http://www.mandt.de/backupallokcom/escience.htm.

Ganrong, X., C. Yue, C. Yun, L. Xiaorong and L. Xing. 2003. Production of monacolin K in solid-state fermentation of *Monascus* sp. 9901 that does not produce citrinin. **Paper presented at the Poster Session of The 1st International Symposium and Workshop on Insight into the World of Indigenous Fermented Foods for Technology Development and Food Safety**. Bangkok, Thailand.

Harntrikanon, N. 2006. **Effect two Thai desserts made from fermented rice powder containing natural food colors derived from *Monascus* spp. on urethane mutagenicity in *Drosophila melanogaster* and their antioxidant activity**. M. S. Thesis, Mahidol University, Bangkok.

Hiroi, T., T. Shima and N. Ogasanara. 1979. Hyperpigment-productive mutant of *Monascus anka* for solid culture. **Agr. Biol. Chem.** 43: 1975-1976.

Jongrungruangchok, S. P. Kittakoop, B. Yongsmith, R. Bavovada, S. Tanasupawat, N. Lartpornmatulee and Y. Thebtaranonth. 2004. Azaphilone pigments from a yellow mutant of the fungus *Monascus kaoliang*. **Phytochem.** 65: 2569-2575.

Kruawan, K., K. Kangsadalampai, B. Yongsmith and T. Sriyapai. 2005. Effect of *Monascus* colourants on the mutagenicity of nitrite treated 1- aminopyrene using Ames test. **Thai J. Pharma. Sci.** 29: 29-41.

Li, Y-g., F. Zhang, Z-t. Wang and Z-b. Hu. 2004. Identification and chemical profiling of monacolins in red yeast rice using high-performance liquid chromatography with photodiode array detector and mass spectrometry. **J. of Pharma. and Biomed. Analy.** 35: 1101–1112.

Ma, J., Y. Li, Q. Ye, J. Li, Y. Hua, D. Ju, D. Zhang, R. Cooper and M. Chang. 2000. Constituents of red yeast rice, a traditional Chinese food and medicine. **J. Agri. Food Chem.** 48: 5220-5225.

Manzoni, M. and M. Rollini. 2002. Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. **Appl. Microbiol. Biotechnol.** 58: 555-564.

Wong, H.C., C. Lin, P.E Koehler. 1981. Regulation of growth and pigmentation of *Monascus purpureus* by carbon and nitrogen concentrations. **Mycologia.** 73:649–654.

Yongsmith, B., L. Chitradon, S. Krairak, W. Tabloka and R. Bavavoda. 1990. Cassava fermentation of yellow pigments and amylolytic enzymes of a mutant of *Monascus* spp. in submerge cultivation. **Microbial Utilization of Renewable Resources** 7: 354-363.

Yongsmith B., V. Kitprechavanich, L. Chitradon, C. Chaisrisook, N. Budda. 2000. Color mutants of *Monascus* sp. KB9 and their comparative glucoamylase on rice solid cultures. **J. of Molec. Cataly. B: Enzymatic** 10: 263-272.

Yongsmith, B., S. Krairak and R. Bavavoda. 1994. Production of yellow pigments in submerged culture on a mutant of *Monascus* spp. **J. Ferment. Bioeng.** 78: 223-228.