Using millet as substrate for efficient production of monacolin K by solid-state fermentation of *Monascus ruber*

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In this study, various grains such as rice, millet, corn, barley and wheat were used as raw materials for monacolin K production by solid-state fermentation of *Monascus ruber*. Among these substrates, millet was found to be the best one for monacolin K production, by which the yield reached 7.12 mg/g. For enhanced monacolin K production, the effects of fermentation time, charge amount, initial moisture content and inoculum volume were systematically investigated in the solid-state fermentation of *M. ruber*. Moreover, complementary carbon source and nitrogen source were added for further improving the production of monacolin K. Results showed that the maximum production of monacolin K (19.81 mg/g) could be obtained at the optimal conditions. Compared with the traditional red mold rice, using millet as substrate is promising for high production of monacolin K in the solid-state fermentation of *M. ruber*.

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[Key words: Monascus; Solid-state fermentation; Monacolin K; Millet; Process optimization]

Cardiovascular disease has been the leading cause of death around the world in recent years. Among various detrimental factors, high cholesterol level in blood is the major one which lead to cardiovascular disease (12). According to the report of the World Health Organization (WHO), raised cholesterol problem is estimated to cause 2.6 million deaths annually, which simultaneously increases the risks of heart disease and stroke.

In the cholesterol biosynthesis pathway, 3-hydro-3-methylglutaryl-coenzyme is the key enzyme which catalyzes the rate-limiting step (3–5). Structurally similar to the substrate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), monacolin K (lovastatin) can compete the key enzyme and hence develop as the most potent drug for hypercholesterolemia treatment and reducing low density lipoprotein cholesterol (1,6,7). Monacolin K can be naturally produced by several fungal species, such as *Monascus* spp., *Aspergillus terreus*, *Penicillium citrinum* and *Paecilomyces viridis* (6–12). Compared to the other fungi, the use of *Monascus* spp. as monacolin K producing strain processes several advantages, such as non-pathogenic and traditionally acted as fermentation food in Asian countries. Both solid-state fermentation (SSF) and submerged fermentation (SMF) can be applied for producing monacolin K by *Monascus* spp. Compared with SMF, SSF offers the advantages of high concentration of the end-product, low water demand, low sterility requirement and low product repression effect (13).

At present, rice has been dominantly applied as substrate in the production of monacolin K by SSF of *Monascus* spp., by which the yields of monacolin K could be up to 16.45 mg/g (14). It is the traditional reason that Asian people still use rice as main substrate in Monascus fermented product. However, in order to explore more unique functional products, it is a promising way to develop the SSF of *Monascus* spp. with different kinds of substrates. The use of other grain, non-grain foodstuff, agricultural residues and inert carrier as substrate has also been reported for producing monacolin K by SSF of *Monascus* spp. (15–19). For example, Lee et al. (15) used sweet potato, potato, cassava, and dioxascara as the substrates to produce monacolin K by *Monascus purpureus* NTU 301. The results show that dioxascara was the best substrate which could produce monacolin K at 2.584 mg/g. Lu et al. (16) used inert carrier bagasse as the substrate to produce monacolin K at 12.9 mg/g, when glycerol and soybean powder were added as the main supplementary nutrients. However, the main problem with non-grain substrates is that the yield of monacolin K is too low and not feasible for industrial application. Comparatively, due to the special features of different grains, they can be utilized by *Monascus* spp. and produce more excellent function.

In this study, a variety of common grains were selected as the substrates for SSF of *Monascus* spp. Afterwards, in order to obtain high yield of monacolin K, supplementary nutrients were added with the optimal substrate. It is promising that this study can broaden the substrate spectrum for SSF of *Monascus* spp., and obtain the suitable SSF conditions applicable to the other substrates.

**MATERIALS AND METHODS**

Chemicals and strain: Lovastatin standard was purchased from China Food and Drug Administration (China). All other chemicals were supplied by National Medicines Corporation Ltd. (China). *Monascus ruber* was obtained from the collection of Key Laboratory of Industrial Biotechnology, Ministry of Education, School of...
Biotechnology, Jiangnan University (China). Cultures of *M. ruber* were periodically maintained on potato dextrose agar (PDA) and then store at 4 °C, after growth on PDA for 8 days at 30 ºC (20,21).

**Preparation of spore suspension and seed culture** A suspension of spores was obtained by washing the actively growing slants with sterile water and adjusted to a concentration of about 1 × 10^8 spores/mL. And 10 mL of spore suspension was added into 500 mL Erlenmeyer flasks containing 100 mL seed culture medium (60 g/L glucose, 25 g/L peptone, 2 g/L NaNO₃, 1 g/L MgSO₄·7H₂O and 1 g/L KH₂PO₄) and incubated at 30 ºC for 2 days on a shaker incubator at 170 rpm (22).

**Solid-state fermentation** Rice, millet, corn, wheat and barley were purchased from the local supermarket of Auchon (Wuxi, China), and used as substrates for SSF of *M. ruber*. The moisture content of the five substrates were measured by a moisture analyzer. Fifty grams of substrates were soaked with appropriate amount of distilled water to control the initial moisture content at 50%, which were put into 500 mL Erlenmeyer flasks and autoclaved at 121 ºC for 30 min. After being cooled, the substrates were inoculated with 10 mL seed culture. SSF was firstly carried out at 30 ºC for 2 days, followed by further incubation at 25 ºC for 18 days (16).

**Monacolin K analysis** After SSF, the products were dried to constant weight at 50 ºC. Monacolin K was extracted from 0.5 g of dry solid cultures which were ground to powder (80 mesh) with 50 mL of 70% ethanol. Extraction of samples was carried out in 55 ºC water bath for 1 h with intermittent shaking. The extract was filtered through a 0.45 µm membrane and then analyzed by high-performance liquid chromatography (HPLC, Waters 1525, Waters, Milford, MA, USA). A ZORBAX SB C-18 column (250 × 4.6 mm, 5 µm) and a mobile phase of acetonitrile-water (55:45, v/v) were used. Detection was carried out by UV detector at 238 nm (23,24).

**Monacolin K content (mg/g)**
\[
\text{c (mg/L) = } \frac{c \times 0.5 \times 100}{\text{ moisture content of product}}
\]

where c is the monacolin K concentration determined in the extract by HPLC (mg/L).

**Experimental design** A traditional single-factor-multiple-level experimental design was applied to study the effect of fermentation time, charge amount, initial water content, inoculation amount, content of carbon source and nitrogen source on monacolin K production by SSF of *M. ruber*. On the basis of single factor experiment, four factors including the inoculation amount, the content of glycerol and soybean powder, and the charge amount of millet were selected for the L₉ (3⁴) orthogonal test (Table 1) (25).

**RESULTS AND DISCUSSION**

**Effect of different substrates** Different grains may possess their own characteristics such as different particle size, water absorption capacity, viscosity, and nutrients, which would be of great importance for the microbial growth and metabolism in SSF (26). In this study, five kinds of typical grains were selected as raw materials to investigate the effect of substrates on the monacolin K production by SSF of *M. ruber*.

As shown in Fig. 1, among the five kinds of grains, millet supported the highest concentration of monacolin K by SSF of *M. ruber* (7.25 mg/g), which was significantly higher than that of the commonly used rice (5.38 mg/g). Comparatively, the concentration of monacolin K was only 2.45 and 2.69 mg/g with wheat and barley, respectively, indicating that they were not suitable substrates for the production of monacolin K by SSF of *M. ruber*. With a thick seed coat, it was difficult for barley and wheat to absorb water, and thus they were not appropriate for further growth of *Monascus* mycelia.

![FIG. 1. Effects of different grains on the production of monacolin K by SSF of *M. ruber*](image)

Venkateswaran et al. (18) also used different substrates for SSF of *M. purpureus* to produced monacolin K, in which the best substrate was finger millet. As shown in Fig. 1, lower content of monacolin K was obtained when using rice and wheat as substrates for SSF. The results were consistent with the phenomenon that less mycelia covered and penetrated the substrate such as wheat and barley during the fermentation process. Compared with other solid substrates, millet has smaller particle size and thus larger specific surface area, which is beneficial for the growth of microorganisms. Moreover, millet is less viscous than rice after autoclaved, which is not easy to agglomerate during the fermentation process.

In order to further investigate the underlying mechanism of the substrate effect, the other grains including rice, corn, wheat and barley were grinded into small millet-like particle size. However, the production of monacolin K with small particle size of these substrates remained lower than that of millet, indicating the particle size was not the critical parameter of the substrate (data not shown). Comparatively, the matrix viscosity seems a more important factor. Because after grinded into smaller particle size, these substrates were more viscous and easily formed large block in the fermentation process, which was not suitable for mycelial growth and thus resulted in lower concentration of monacolin K. Starch is one of the most important composition of these grains and it has been indicated that there is a close relationship between amylose content and the substrate viscosity. The higher the amylose content is, the lower the viscosity is. Hence, the amylose content of two typical substrate rice and millet were measured and compared. Results showed that the amylose content of millet (23.6%) was higher than that of rice (19.1%), suggesting that the viscosity of rice was higher than that of millet. By observing the substrate morphology during the solid-state fermentation process, millet was not easy to agglomerate due to its high amylose content, which was beneficial for the biosynthesis of monacolin K. Further in-depth investigation at molecular level are underway to provide more evidence for this study. Therefore, millet was chosen as the suitable substrate for producing monacolin K in the subsequent experiments.

**Time course of SSF** In order to make rational use of time and resources, a time course study on the production of monacolin K was necessary. As shown in Fig. 2, the concentration of monacolin K increased by consumption of substrate along with the fermentation

**TABLE 1. Effects of four factors (inoculation amount, glycerol content, soybean powder and charge amount) at three levels on monacolin K production of *Monascus ruber***

<table>
<thead>
<tr>
<th>Level</th>
<th>Inoculation amount (%)</th>
<th>Glycerol content (%)</th>
<th>Soybean powder content (%)</th>
<th>Charge amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>15</td>
<td>2.5</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>20</td>
<td>3</td>
<td>50</td>
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<td>3</td>
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<td>70</td>
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