

# ***Monascus* Mutant Strain and Method for Preparing Red Yeast Rice or Monacolin K**

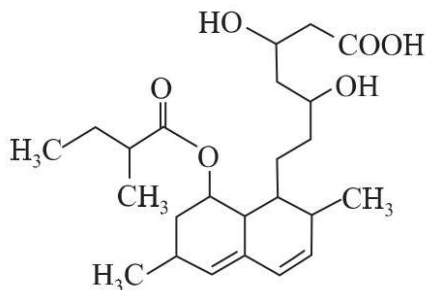
## **Technical Field**

The present invention belongs to the field of biomedicine and fermentation engineering, and relates to a *Monascus* mutant strain and a method for preparing red yeast rice or monacolin K. The present invention also relates to red yeast rice prepared by the *Monascus* mutant strain.

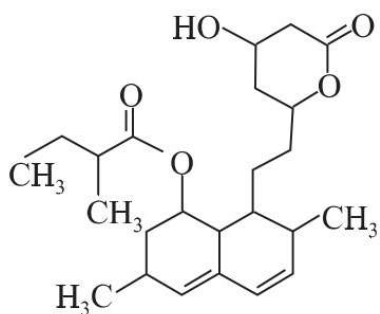
## **Background Art**

Red yeast rice is made from rice as raw material via inoculation with *Monascus purpureus* and fermentation in solid state, and also known as Fermentum Rubrum. Although red yeast rice can be used in the fields of pigments, food additives, health foods, and medicines, the ingredients contained therein vary greatly for different uses. When red yeast rice is used as a food additive, it can be added directly or made into monascus red pigment or monascus yellow pigment and then added, and it does not contain or may contain only trace amount of lovastatin. Modern research shows that the fermentation products of medicinal red yeast rice contain ingredients such as monacolin K, ergosterol, and monascus pigments.

Monacolin K is the main active ingredient in fermentation products of red yeast rice. Monacolin K exists in two forms: acidic/open-ring (called acidic monacolin K or lovastatin acid, referred to as MKA in the present invention) and lactone/closed-ring (called lactone monacolin K or lovastatin, referred to as MK in the present invention). The lactone monacolin K is more stable than the acidic monacolin K. Under acidic conditions, the acidic monacolin K will continuously transform into the lactone form. At present, the marketed drug lovastatin has the activity of lowering blood lipids and cholesterol. Lovastatin is an inactive lactone prodrug and must be converted to lovastatin acid by chemical or enzymatic conversion to exert its effect. The structural formulas of acidic monacolin K (referred to as "MKA") and lactone monacolin K (referred to as "MK") are shown in Formula I and Formula II below, respectively.



Formula I

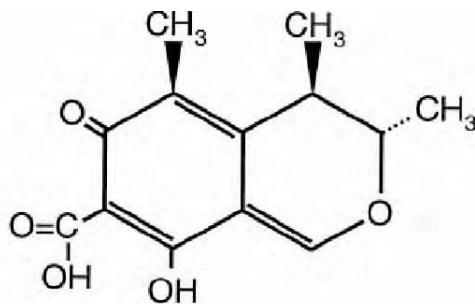


Formula II

Hydroxymethylglutaryl CoA (HMG-CoA) reductase is the rate-limiting enzyme for cholesterol biosynthesis in the human body, and lovastatin can competitively bind to the HMG-CoA reductase receptor, thereby inhibiting the activity of HMG-CoA reductase and further reducing the synthesis of cholesterol. At the same time, lovastatin can also promote the conversion of cholesterol into bile and excretion from the body by increasing the number, activity and affinity of low-density lipoprotein receptors on the liver cell membrane, thereby reducing the cholesterol content in plasma.

In the fermentation of red yeast rice, the production of citrinin is a common phenomenon, and its detection limit and quantification limit in red yeast rice and products thereof stipulated in the national standard of China are 25 $\mu$ g/kg and 80 $\mu$ g/kg, respectively, and its quantification limit in red yeast rice in EU is 100 $\mu$ g/kg. Some studies have reported that the content of citrinin in red yeast rice is 1.40 to 24.70  $\mu$ g/g (Screening of monascus strains with high monacolin K production and low citrinin production and optimization of their fermentation conditions, Zhuang Yue'e; Chen Huaguan, *Fujian Agricultural Science and Technology*, 2020-01-28).

Citrinin is a mycotoxin produced by the growth and metabolism of *Penicillium* or *Aspergillus* (e.g., *Monascus*). Citrinin is a lemon yellow, disordered crystal structure with a melting point of 175°C. Under dry conditions, citrinin decomposes when the temperature exceeds 175°C, and in aqueous solution, citrinin decomposes when the temperature exceeds 100°C. The structural formula of citrinin is shown in Formula III below.



Formula III

Citrinin has strong renal toxicity. Toxicological experiments have shown that citrinin has neurotoxicity, nephrotoxicity and hepatotoxicity, can induce cell mutations and lead to malignant tumors, can cause kidney enlargement, tubular dilatation, epithelial cell necrosis and central hemorrhage in the human body, and has strong teratogenicity and carcinogenicity. In addition, some studies have found that citrinin can reduce the secretion of dopamine in the brain of animals, leading to an increase in the concentration of some neurotransmitters, which causes great harm to the human body. These reasons greatly limit the use of red yeast rice products. The national standards of China establish very strict limits on the content of citrinin in red yeast rice products.

At present, it is still in need to develop new monascus strains and processes for preparing red yeast rice.

### **Contents of the present invention**

After in-depth research and creative work, the inventors of the present invention screened out three monascus mutant strains M7, M8 and M2-4 with high yield of monacolin K by utilizing ultraviolet or composite mutagenesis. Among them, after solid-state fermentation with M7 and M8 for 25 days, the total amount of monacolin K was detected to be 12.42 mg/g and 12.49 mg/g, respectively, which was 26% and 27% higher than that of the control group, respectively. When mutagenic strains M7 and M8 were co-cultured with *Saccharomyces cerevisiae*, it was found that after adding 3% inactivated yeast fermentation broth, the highest total amount of monacolin K in the fermented red yeast rice was 10.14 mg/g, which was 11.31% higher than that of the control group. The total amount of monacolin K in the red yeast rice fermented with M2-4 was 13.21 mg/g. No citrinin was detected in the red yeast rice prepared by the monascus mutant strains of the present invention at the detection limit of 25 µg/kg and even 5 µg/kg.

Therefore, the following invention is provided:

One aspect of the present invention relates to a monascus mutant strain,

which is deposited in the China General Microbiological Culture Collection Center (CGMCC) with an accession number of CGMCC No. 40403 (corresponding to the strain M7 of the present invention), or

which is deposited in the China General Microbiological Culture Collection Center (CGMCC) with an accession number of CGMCC No. 40487 (corresponding to the strain M2-4 of the present invention).

The above two strains of the present invention belong to the genus *Monascus*, and are classified as *Monascus purpureus* in further classification. Considering the similarity between strains to a certain extent, if the above two strains are classified as *Monascus ruber* from the

morphological characteristics and/or cultural characteristics of the strains, they are within the scope of protection of the present invention as well.

Another aspect of the present invention relates to a monascus fermentation product obtained by fermentation with the *Monascus* mutant strain of the present invention;

preferably, the monascus fermentation product is a monascus fermentation broth obtained by fermentation with the *Monascus* mutant strain of the present invention in a liquid fermentation medium.

In some embodiments of the present invention, in the monascus fermented product,

the liquid fermentation medium comprises the following components:

30-150 g/L glucose, 2-30 g/L peptone, 0.5-7 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.01-1.2 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01-0.8 g/L  $\text{CaCl}_2$ ; prepared with potato juice or distilled water;

65-95 g/L glucose, 5-16 g/L peptone, 1-3 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.2-0.8 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05-0.2 g/L  $\text{CaCl}_2$ ; prepared with potato juice or distilled water; or

80 g/L glucose, 10 g/L peptone, 2 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.5 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g/L  $\text{CaCl}_2$ ; prepared potato juice or distilled water to obtain the fermentation medium.

In some embodiments of the present invention, in the monascus fermentation product,

the fermentation temperature is 25°C to 35°C, 28°C to 32°C, or 30°C; and/or

the fermentation time is 20-72 hours, 24-50 hours, 36-48 hours, or 48 hours.

Another aspect of the present invention relates to a fermentation broth combination, comprising:

a yeast fermentation broth; and

the monascus fermentation broth described in any one of the present invention,

wherein the yeast fermentation broth and the monascus fermentation broth are mixed or not mixed.

In some embodiments of the present invention, in the fermentation broth combination, the yeast fermentation broth is a fermentation broth of *Saccharomyces cerevisiae* or a fermentation broth of beer yeast;

optionally, the yeast fermentation broth is an inactivated yeast fermentation broth or an uninactivated yeast fermentation broth.

In some embodiments of the present invention, in the fermentation broth combination, the yeast fermentation broth is obtained by a method comprising the following conditions:

a yeast is activated in a wort, preferably in a wort of 12 Brix;  
the activation temperature is 25°C to 30°C, preferably 28°C; and/or  
the activation time is 12-36 hours, preferably 24 hours.

Another aspect of the present invention relates to a method for preparing red yeast rice, comprising the following steps:

inoculating the monascus mutant strain of the present invention, the monascus fermentation product of any one of the items of the present invention, or the fermentation broth combination of any one of the items of the present invention into a rice culture medium for cultivation to obtain a culture product, namely, red yeast rice;

optionally, further comprising a step of drying and/or crushing the culture product.

In some embodiments of the present invention, in the method for preparing red yeast rice, the inoculation ratio is that:

the yeast fermentation broth is 1% to 5%, 2% to 4% or 3% of the mass of the rice culture medium; and/or

the monascus fermentation broth or the fermentation broth combination is 15% to 40%, 20% to 25%, 18% to 23% or 20% of the mass of the rice culture medium.

In the present invention, unless otherwise specified, the inoculation ratio refers to the volume of the fermentation broth/the mass of the rice culture medium (mL/g).

In some embodiments of the present invention, in the method for preparing red yeast rice:

the rice culture medium comprises rice flour, bran, peptone, glucose and water;

preferably, the rice culture medium comprises the following components expressed in mass percentage:

35% to 42% rice flour, 6% to 9% bran, 1.2% to 2.2% peptone, 1.4% to 3.0% glucose and 48% to 52% water; for example:

38.5% rice flour, 7.5% bran, 1.5% peptone, 2.5% glucose and 50% water;

37.7% rice flour, 7.5% bran, 1.9% peptone, 2.6% glucose and 50.2% water;

41.1% rice flour, 6.2% bran, 1.2% peptone, 1.4% glucose and 50.1% water; or

35.8% rice flour, 8.9% bran, 2.1% peptone, 3.0% glucose and 50.1% water.

In some embodiments of the present invention, in the method for preparing red yeast rice, the rice culture medium is composed of the above components.

In some embodiments of the present invention, in the method for preparing red yeast rice, the rice culture medium is sterilized, for example, by high temperature sterilization.

In some embodiments of the present invention, the method for preparing red yeast rice is characterized by any one, two or all three of the following items (1) to (3):

(1) the culture time is 15-38 days, 18-32 days, 20-25 days, 22-30 days, 24-26 days or 25 days;

(2) the culture temperature is 18°C to 35°C, 28°C to 32°C, 28 to 30°C, or 30°C;

(3) the pH is adjusted to an appropriate value, for example, the pH value is 3 to 7, 4 to 6.5 or 5 to 6.

Another aspect of the present invention relates to a red yeast rice, which is prepared by the monascus mutant strain of the present invention; preferably, it is prepared by the method for preparing red yeast rice as described in any one of items of the present invention.

In some embodiments of the present invention, the red yeast rice is characterized in that:

it does not contain citrinin, or the content of citrinin is less than 25 µg/kg or less than 5 µg/kg; and/or

it does not contain aflatoxin B1, or the content of aflatoxin B1 is less than 5 ppb.

In some embodiments of the present invention, the content of monacolin K in the red yeast rice is greater than or equal to 8.9 mg/g, greater than or equal to 9.97 mg/g, greater than or equal to 12.42 mg/g, greater than or equal to 12.85 mg/g, or greater than or equal to 13.21 mg/g;

preferably, the content of acidic monacolin K in the red yeast rice is greater than or equal to 1.6 mg/g, greater than or equal to 1.8 mg/g, greater than or equal to 2.0 mg/g, greater than or equal to 2.2 mg/g, greater than or equal to 2.4 mg/g, greater than or equal to 2.5 mg/g, greater than or equal to 3.0 mg/g, greater than or equal to 3.5 mg/g, greater than or equal to 3.9 mg/g, greater than or equal to 4.5 mg/g, or greater than or equal to 5.2 mg/g.

In some embodiments of the present invention, for the content of acidic monacolin K in the red yeast rice: the content of lactone monacolin K is at a high proportion, for example, the proportion is  $\geq 0.20$ ,  $\geq 0.21$ ,  $\geq 0.22$ ,  $\geq 0.23$ ,  $\geq 0.24$ ,  $\geq 0.25$ ,  $\geq 0.26$ ,  $\geq 0.27$ ,  $\geq 0.28$ ,  $\geq 0.29$ ,  $\geq 0.30$ ,  $\geq 0.31$  or  $\geq 0.32$ .

Another aspect of the present invention relates to an extract of red yeast rice, which is obtained by extracting the red yeast rice as described in any one of the items of the present invention with ethanol or water.

In some embodiments of the present invention, the red yeast rice as described in any one of the items of the present invention or the extract of red yeast rice as described in any one of the items of the present invention is used for treating or preventing hyperlipidemia or osteoporosis.

Another aspect of the present invention relates to a method for preparing monacolin K, comprising the step of isolating monacolin K from the red yeast rice as described in any one of the items of the present invention or the extract of red yeast rice of the present invention;

preferably, the monacolin K is lactone monacolin K and/or acid monacolin K.

MKA refers to acidic monacolin K, MK refers to lactone monacolin K, and MK+MKA refers to the total amount of monacolin K.

In the present invention, unless otherwise specified, the term "monacolin K content" refers to the total content of acidic monacolin K and lactone monacolin K.

Another aspect of the present invention relates to a use of the red yeast rice or the extract of red yeast rice as described in any one of the items of the present invention in the preparation of a medicament or health food for treating or preventing hyperlipidemia or osteoporosis.

Another aspect of the present invention relates to a method for treating or preventing hyperlipidemia or osteoporosis, comprising a step of administering to a subject in need thereof an effective amount of the red yeast rice or the extract of red yeast rice as described in any one of the items of the present invention.

The term "effective amount" refers to a dose that can achieve treatment, prevention, alleviation and/or relief of the disease or condition as described in the present invention in a subject.

The term "disease and/or condition" refers to a physical state of the subject, which is related to the disease and/or condition as described in the present invention.

The term "subject" may refer to a patient or other animal, especially a mammal, such as human, dog, monkey, cattle, horse, etc., which receives the medicament or pharmaceutical composition of the present invention to treat, prevent, alleviate and/or relieve the disease or condition as described in the present invention.

Beneficial effects of the present invention:

The present invention has achieved one or more of the following technical effects:

(1) The monascus mutant strain of the present invention can increase the yield of monacolin K whether it is fermented and cultured alone or co-cultured with *Saccharomyces cerevisiae*;

(2) The content of citrinin in the red yeast rice produced by the present invention is below 25 µg/kg, or even below 5 µg/kg;

(3) In the red yeast rice produced by the present invention, aflatoxin B1 is not detected or has a content less than 5 ppb;

(4) The red yeast rice obtained by fermentation of the monascus mutant strain of the present invention has a high total amount of monacolin K, especially a high content of acidic monacolin K;

(5) The red yeast rice of the present invention contains a plurality of active ingredients, including multiple kinds of statins, sterols, pigments, etc., especially  $\gamma$ -aminobutyric acid, ergosterol, etc.

(6) The monascus mutant strain of the present invention has good stability, thereby effectively solving some common problems in the prior art, such as:

the activity of the strain declines after multiple generations of subculture;

the content of active ingredients decreases after fermentation; or

after prolonged and repeated use of a single mutagen, the strain develops a "fatigue effect" toward the mutagen.

#### **Brief Description of the Drawings**

Figure 1 shows the effect of UV irradiation time on the lethality of *Monascus* MY-11.

Figure 2A shows the colony morphology on malt agar medium.

Figure 2B shows the morphology of *Monascus* M7.

Figure 2C shows the morphology of *Monascus* M7.

Figure 2D shows the morphology of *Monascus* M7.

Among which, Figure 2B, Figure 2C and Figure 2D show the growth status of the same sample in different view fields, respectively.

Figure 3 shows the chromatogram of the citrinin control solution (20.0 ng/ml).

Figure 4 shows the chromatogram of citrinin detected in *Monascus* M7.

Biological materials involved in the deposit:

*Monascus purpureus* M7, which is deposited in the China General Microbiological Culture Collection Center (CGMCC) on November 2, 2022, with an accession number of CGMCC No. 40403, and the deposit address is No. 3, Yard 1, Beichen West Road, Chaoyang District, Beijing, Institute of Microbiology, Chinese Academy of Sciences, Postal Code: 100101.

*Monascus purpureus* M2-4, which is deposited in the China General Microbiological Culture Collection Center (CGMCC) on January 13, 2023, with an accession number of CGMCC No. 40487, and the deposit address is No. 3, Yard 1, Beichen West Road, Chaoyang District, Beijing, Institute of Microbiology, Chinese Academy of Sciences, Postal Code: 100101.

### **Specific Models for Carrying Out the present invention**

The following will describe the embodiments of the present invention in detail in conjunction with the examples, but those skilled in the art will understand that the following examples are only used to illustrate the present invention and should not be regarded as limiting the scope of the present invention. If the specific conditions were not specified in the examples, the conventional conditions or the conditions recommended by the manufacturer were followed. If the manufacturer of the reagents or instruments used was not specified, they were all conventional products that can be purchased commercially.

#### **Materials and reagents**

*Monascus purpureus* MY-11: a strain preserved in our laboratory;

*Saccharomyces cerevisiae* 2.2084: obtained from the China General Microbiological Culture Collection Center;

Glucose, magnesium sulfate heptahydrate, anhydrous calcium chloride, ammonium dihydrogen phosphate, peptone (all analytical grade or biochemical reagents);

Rice flour, potatoes, bran: commercially available;

Methanol, formic acid and ammonium formate: all chromatographically pure;

95% ethanol (analytical grade): purchased from Sinopharm Chemical Reagent Company;

Lovastatin (lactone monacolin K) as control: commercially available, content: 99.7%;

Water: ultrapure water;

Lovastatin acid sodium salt (acid monacolin K) as control: commercially available, purity: 98%;

Potato dextrose agar medium (PDA medium for short): 46.0 g of the same was taken, added to 1000 mL of distilled water, and sterilized at 121°C for 30 min for later use. The agar slant culture-medium mentioned in the following examples was PDA medium.

Liquid fermentation medium: the liquid fermentation medium was prepared by taking glucose at 80 g/L, peptone at 10 g/L,  $\text{NH}_4\text{H}_2\text{PO}_4$  at 2 g/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  at 0.5 g/L and  $\text{CaCl}_2$  at 0.1 g/L, and dissolving them in potato juice instead of distilled water for later use.

Rice medium: rice flour 38.5%, bran 7.5%, water 50%, glucose 2.5%, peptone 1.5%, pH value 5, sterilized at 121 °C for 20 min.

#### Instruments and devices

ZHWY-2102 constant temperature culture oscillator: Shanghai Zhicheng Co., Ltd.;

LRH-250-HSE constant temperature and humidity incubator: Zhujiang Taihongjun Instrument;

BXM-110VE vertical pressure steam sterilizer: Boxun;

WGL-230B electric heating blast drying oven: Tianjin Taisite Instrument Co., Ltd.;

FW80 high-speed universal crusher: Tianjin Taisite Instrument Co., Ltd.;

Shimadzu LC-20AT high-performance liquid chromatograph;

KQ5200DE ultrasonic cleaning machine: Kunshan Hechuang Ultrasonic Instrument Co., Ltd.;

METTLER XPE205DR electronic balance;

Ultrapure water instrument: PALL, USA.

#### Preparation Example 1: Acquisition of *monascus* mutant strain

Preparation of spore suspension: A strain (*Monascus purpureus* MY-11) growing on agar slant culture-medium was taken, and added with a small amount of sterile water; the spores on the slant were scraped with an inoculation loop, added into a triangular flask and shaken for 15 minutes to make the spores fully dispersed; then a funnel with 4 layers of lens paper was used to filter out mycelia; and the volume was adjusted to 50mL to obtain a spore suspension.

Laboratory test on the growth state of the strain were carried out with the initially screened ultraviolet lamp wattage values (5, 10, 15, 20 w) and distances from ultraviolet lamp (15, 30, and 45 cm). It was found that better effects were achieved when the wattage value was 15 w and the distance was 30 cm.

Ultraviolet mutagenesis: 10mL of the spore suspension was taken and added into a sterile culture dish, placed 30cm under a 15W ultraviolet lamp, and irradiated for 0s, 10s, 20s, 30s, 40s, and 60s, respectively. 0.5mL of the spore suspension at each irradiation time period was taken after mutagenesis, and diluted in a 10-fold series to reach concentration of  $10^{-4}$ . 1mL of each dilution was taken and coated on PDA medium, and cultured at 30°C in the dark for 7 days. The number of colonies on the plate were recorded, and the lethality of mutagenesis was calculated.

Lethality/% = (number of colonies grown on the control plate - number of colonies grown on the mutagenesis plate)/number of colonies grown on the control plate.

Initial screening of monascus: the colonies grown on the plate were observed, the strains with faster growth or changed morphological and color were picked, inoculated PDA medium and cultured at 30°C.

Rescreening of monascus: the screened monascus strains were inoculated at 5% (volume of slant strain suspension/volume of liquid culture medium) into liquid fermentation medium, and cultured on a shaker at 30°C for 2 days. The liquid strains were pipetted and inoculated rice culture medium, stirred and mixed, and cultured at 30°C for 25 days to obtain red yeast rice products.

The red yeast rice products were taken and subjected to monacolin K detection to select mutagenic strains with high monacolin K content. The detection method was as follows:

The red yeast rice products were dried at 90°C for 24h and crushed into red yeast rice powders by a high-energy grinder. The contents of MK and MKA in the red yeast rice powders were detected. The specific detection method was carried out according to the literature (Li Xuemei, et al. Determination of contents of lovastatin and lovastatin acid in Xuezhikang capsules by HPLC [J]. Chinese Pharmacist, 2012, 15(02)).

The results are shown in Figure 1.

As shown in Figure 1, when the dose for ultraviolet mutagenesis reached 20s, the lethality of monascus reached 76%, so the optimal dose for ultraviolet mutagenesis was determined as 20s.

The *Monascus* MY-11 was subjected to ultraviolet mutagenesis, and the lethality rate was used to investigate the damaging effect of ultraviolet rays on monascus. In order to obtain a higher positive mutation rate and facilitate the screening of mutagenic strains, the optimal lethality was selected between 70% and 80%. Without being bound by any theory, in order to improve the success rate of mutagenesis, plate colonies with higher lethality rate should be selected for subsequent experiments. However, mutant strains on plates with too high lethality rate were prone to reverse mutation and had unstable properties. Therefore, in actual operation, the dose for mutagenesis on plates with a lethality rate of 70% to 80% was generally selected as the optimal dose for mutagenesis.

Ultraviolet mutagenesis was carried out with *Monascus purpureus* MY-11 as the original strain according to the above dose for ultraviolet mutagenesis. After initial screening of colony morphology, 8 ultraviolet mutagenic strains were obtained, which were numbered M1 to M8, respectively. They were subjected to solid-state fermentation according to the above method, and their contents of monacolin K were determined. It was checked whether the contents were higher

than the content of monacolin K in the original strain, and those with higher content than that of the original strain were determined as positive mutation. The results are shown in Table 1.

As shown in Table 1, compared with the original strain *Monascus purpureus* MY-11, the monacolin K contents of the mutagenic strains M7 and M8 had increased significantly, reaching 12.42 mg/g (referring to the content of the dried fermentation product of monascus, the same below) and 12.49 mg/g, respectively; which had increased by 26% and 27% as compared with the original strain MY-11, respectively; and they would be used as the strains for the next co-culture.

Table 1: Monacolin K contents detected for rescreening ultraviolet mutagenic strains of *Monascus* MY-11

| Sample No. | MKA (mg/g) | MK (mg/g) | MK+MKA (mg/g) | Increase rate, % |
|------------|------------|-----------|---------------|------------------|
| MY-11      | 2.59       | 7.25      | 9.84          | 0                |
| M1         | 2.85       | 6.22      | 9.07          | -8%              |
| M2         | 2.76       | 8.27      | 11.03         | 12%              |
| M3         | 2.50       | 7.71      | 10.21         | 4%               |
| M4         | 2.96       | 7.44      | 10.4          | 6%               |
| M5         | 2.63       | 6.08      | 8.71          | -11%             |
| M6         | 2.99       | 6.59      | 9.58          | -3%              |
| M7         | 2.47       | 9.95      | 12.42         | 26%              |
| M8         | 3.24       | 9.25      | 12.49         | 27%              |

Note: MKA refers to acidic monacolin K, MK refers to lactone monacolin K, and MK+MKA refers to the total amount of monacolin K.

M7 and M8 were identified as *Monascus purpureus* by microbiological identification, and M7 was sent to a strain identification agency for identification. After the strain was subjected to identification of culture characteristics, microscopic characteristics, and rRNA gene sequence, the results showed that the monascus mutant strain of the present invention belonged to *Monascus purpureus*.

The results of rRNA gene sequence determination are as follows, including 18 rRNA fragments, the full sequences of ITS1, 5.8S rRNA, ITS2, and the sequence fragments of 28S rRNA region.

5'-

ATCATTACCGAGTGCGGGTCCCCCTTCGTGGGACCCAACCTCCCACCCGTGATTATTG

TACCTCCTGTTGCTTCGGCGCGGCCCCCGGGGCCCCGCCGGAGACATCTTCTCGAAC  
GCTGTCTTTGAAAAGGATTGCTGTCTGAGTAAACATACCAAATCGGTAAAACTTTC  
AACAAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT  
AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCC  
TGGTATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTACTGCCCCCTCAAGCGCGGCTT  
GTGTGTTGGGCCGCCGTCCCCTGCGCCTCCGGGCAAGGGGGACGGGGCCCGAAAGGC  
AGTGGCGGGCGCCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCAGTAG  
GTCGGGGCCGGGGCCTTTGCCCTCTCCAACCTTTTTTTCCTTAGGTTGACCTCGGATCA  
GGTAGGGATACCCGCTGAACTTAAGCATATCAA -3' (SEQ ID NO: 1)

The colony morphology and microscopic characteristics of *Monascus* M7 are shown in Figures 2A to 2D.

*Monascus* M7 grew fast on malt agar medium. After 7 days of cultivation at 25°C in the dark, the colony diameter was 35mm to 40mm, the aerial hyphae were luxuriant (Figure 2A), and the color was light orange; the back of the colony was dark orange, and the pigment was soluble in the culture medium.

Figures 2B, 2C, and 2D all show *Monascus* M7 under microscope, indicating that conidia were produced in large quantities, the conidiophores were not obviously specialized, straight or slightly curved, and the conidia were spherical, colorless, with slightly rough walls, truncated at the base, and in series, 5.8µm to 9.6µm. No sexual spore-producing structures and spores were found.

#### Experimental Example 1: Genetic stability experiment on monacolin K production by mutagenic strains M7 and M8

The high-yield mutant strains obtained by mutagenic treatment could have unstable genetic traits, and could be prone to back mutations or decline or even disappearance of mutant traits, resulting in problems such as decreased ability to produce monacolin K. Therefore, it was necessary to verify their stability in generation, so as to consider their use method or cycle and the application of strains in industrial production. The mutant strains M7 and M8 with high-yield of monacolin K were inoculated onto agar slant culture-medium, respectively, and subcultured for consecutive 5 generations. The fermentation culture was carried out under the same conditions. The monacolin K content was determined by the aforementioned HPLC method in each generation, and the stability of the mutant strains was analyzed. The results are shown in Table 2.

Table 2: Monacolin K production of mutant monascus strains

| Strain name        | Number of generations | MKA/(mg/g) | MK/(mg/g) | MK+MKA/(mg/g) | Increase rate/% |
|--------------------|-----------------------|------------|-----------|---------------|-----------------|
| <i>Monascus</i> M7 | First generation      | 2.47       | 9.95      | 12.42         | 0               |
|                    | Second generation     | 2.19       | 8.00      | 10.19         | -18%            |
|                    | Third generation      | 2.34       | 7.34      | 9.67          | -22%            |
|                    | Fourth generation     | 1.33       | 7.13      | 8.46          | -32%            |
|                    | Fifth generation      | 1.67       | 7.44      | 9.11          | -27%            |
| <i>Monascus</i> M8 | First generation      | 3.24       | 9.25      | 12.49         | 0               |
|                    | Second generation     | 2.47       | 5.14      | 7.61          | -39%            |
|                    | Third generation      | 2.02       | 6.30      | 8.32          | -33%            |
|                    | Fourth generation     | 1.47       | 7.12      | 8.59          | -31%            |
|                    | Fifth generation      | 1.41       | 6.27      | 7.68          | -39%            |

Note: MKA refers to acidic monacolin K, MK refers to lactone monacolin K, and MK+MKA refers to the total amount of monacolin K.

As shown in Table 2, the mutagenic strains M7 and M8 were passaged 5 times. Compared with the first generation, the total amount of monacolin K in the last 4 generations was lower than that in the first generation, and showed a downward trend, but the content in the fifth generation had stabilized. The content of M7 in the fifth generation increased and was higher than that in the fourth generation, and the content had stabilized.

#### Experimental Example 2: Effect of co-culture on content of monacolin K

*Saccharomyces cerevisiae* 2.2084 was activated in a wort of 12 Brix at 28°C for 24h until the culture medium (the culture medium here referred to wort, which was adjusted to 12 Brix. with a saccharimeter) became turbid, and the yeast fermentation broth was obtained.

The mutagenic strains M7 and M8 were inoculated into the liquid fermentation medium at 5% (V/V), and cultured at 30°C for 48h to obtain monascus fermentation broths.

The yeast fermentation broth and the monascus fermentation broth were inoculated into rice culture medium, and 20% (according to the rice culture medium, V/m, mL/g, the same below) of the monascus fermentation broth were co-cultured with 0, 1%, 2%, 3%, and 4% (according to the rice culture medium, V/m, mL/g, the same below) of the yeast fermentation broth, respectively, stirred and mixed, and cultured at 30°C for 25 days to obtain the fermentation product, namely red

yeast rice. The red yeast rice could be whole rice or the like, or irregular particles, or sometimes powder, which is also called *Fermentum Rubrum*.

The contents of monacolin K in red yeast rice prepared by co-culturing *Monascus* M7 with different amounts of the yeast fermentation broth for 25 days are shown in Table 3. By the addition of 3% the yeast fermentation broth, the highest total content of monacolin K was 9.35 mg/g, which was 2.63% higher than that without addition of the yeast fermentation broth.

Table 3: Monacolin K contents in red yeast rice prepared by co-culturing *Monascus* M7 with yeast for 25 days

| Sample batch No. | Yeast liquid percentage/% | MKA (mg/g) | MK (mg/g) | MK+MKA (mg/g) | Increase rate/% |
|------------------|---------------------------|------------|-----------|---------------|-----------------|
| M7               | 0                         | 1.67       | 7.44      | 9.11          | 0               |
| M7-1             | 1                         | 1.83       | 6.31      | 8.14          | -10.65          |
| M7-2             | 2                         | 1.55       | 5.41      | 6.96          | -23.60          |
| M7-3             | 3                         | 1.74       | 7.61      | 9.35          | 2.63            |
| M7-4             | 4                         | 2.13       | 7.01      | 9.14          | 0.33            |

Note: MKA refers to acidic monacolin K, MK refers to lactone monacolin K, MK+MKA refers to the total amount of monacolin K.

Table 4: Monacolin K contents in red yeast rice prepared by co-culturing *Monascus* M8 with yeast for 25 days

| Sample batch No. | Yeast liquid percentage/% | MKA (mg/g) | MK (mg/g) | MK+MKA (mg/g) | Increase rate/% |
|------------------|---------------------------|------------|-----------|---------------|-----------------|
| M8               | 0                         | 1.41       | 6.27      | 7.68          | 0               |
| M8-1             | 1                         | 1.73       | 4.76      | 6.49          | -15.49          |
| M8-2             | 2                         | 1.94       | 5.63      | 7.57          | -1.43           |
| M8-3             | 3                         | 2.21       | 6.73      | 8.94          | 16.41           |
| M8-4             | 4                         | 1.55       | 5.15      | 6.70          | -12.76          |

Note: MKA refers to acidic monacolin K, MK refers to lactone monacolin K, and MK+MKA refers to the total amount of monacolin K.

The contents of monacolin K in red yeast rice prepared by co-culturing *Monascus* M8 with different amounts of the yeast fermentation broth for 25 days are shown in Table 4. By the addition

of 3% the yeast fermentation broth, the highest total content of monacolin K was 8.94 mg/g, which was 16.41% higher than that without addition of the yeast fermentation broth.

In addition, the inventors also studied the effect of adding inactivated yeast fermentation broth on the strains and monacolin content.

Inactivated yeast fermentation broth: *Saccharomyces cerevisiae* was activated in a wort of 12 Bx, and activated at 28°C for 24 hours until the culture medium became turbid to obtain yeast broth. The yeast broth was sterilized at 100°C for 20 min to obtain the inactivated yeast fermentation broth.

The yeast fermentation broth and monascus fermentation broth were inoculated into rice culture medium, and 20% (based on rice culture medium) of monascus fermentation broth was cultured with 0, 1%, 2%, 3%, and 4% (based on rice culture medium) of the inactivated yeast fermentation broth, respectively, stirred and mixed. The samples were taken after culturing at 30°C for 25 days and 31 days, respectively.

The results are shown in Table 5 below.

Table 5: Monacolin K contents in red yeast rice prepared by culturing *Monascus* M7 with inactivated yeast fermentation broth for 25 days

| Sample batch No. | Inactivated yeast fermentation broth inoculation amount/% | MKA (mg/g) | MK (mg/g) | MK+MKA (mg/g) | Increase rate/% |
|------------------|-----------------------------------------------------------|------------|-----------|---------------|-----------------|
| M7               | 0                                                         | 1.67       | 7.44      | 9.11          | 0.00            |
| M7-1             | 1                                                         | 2.09       | 7.58      | 9.67          | 6.15            |
| M7-2             | 2                                                         | 1.86       | 7.69      | 9.55          | 4.83            |
| M7-3             | 3                                                         | 1.89       | 8.25      | 10.14         | 11.31           |
| M7-4             | 4                                                         | 1.75       | 7.05      | 8.80          | -3.40           |

Note: MKA refers to acidic monacolin K, MK refers to lactone monacolin K, and MK+MKA refers to the total amount of monacolin K.

The effect of MK and MKA contents after culturing *Monascus* M7 (control) with different amounts of the inactivated yeast fermentation broth are shown in Table 5. After 25 days of fermentation, the total amount of monacolin K increased after adding 1%, 2%, and 3% of the inactivated yeast fermentation broth. In particular, by adding 3% of the inactivated yeast fermentation broth, the total amount of monacolin K in the red yeast rice reached 10.14 mg/g, which was 11.31% higher than that of the control M7.

The content determination of the present invention usually refers to the determination result of the red yeast rice obtained after fermentation.

MKA refers to acidic monacolin K, and MK refers to lactone monacolin K.

#### Preparation Example 2: Acquisition and stability test of *Monascus* mutant strains

After long-term repeated use of a single mutagen, the strain was prone to produce a mutagen "fatigue effect", which caused the growth cycle of the strain to be extended and the active metabolites to be reduced. In order to improve the mutagenesis effect, a composite mutagenesis operation is often used in practice. Composite mutagenesis refers to the use of two or more mutagens to treat the starting strain alternately or simultaneously. Since different mutagens have different specific action sites, they cause multiple variations in the genetic material of bacteria and improve the mutagenesis effect.

The concentration of LiCl (0.2‰, 0.4‰, 0.6‰, 0.8‰, 1‰) for mutagenesis was screened by colony morphology and monacolin K content, and the concentration of 0.8‰ was selected after comprehensive comparison.

Bacterial strain: M2; PDA medium containing 0.8‰ LiCl was applied, and the strain M2 selected by ultraviolet mutagenesis was inoculated into the medium containing 0.8‰ LiCl, and cultured in the dark at 30°C for 3 days, and a single colony was picked.

The composite mutagenesis strain was taken, and subcultured for consecutive 5 generations to perform stability study. Inoculation amount was 60 ml for 300g of wet material per bottle. Samples were taken after culturing for 21 and 25 days. The samples were inactivated at 120°C for 50 minutes in a steam sterilizer and dried at 90°C for 24 hours, and the contents of MK and MKA were detected at 21 and 25 days for stability.

*Monascus* M2-4 was cultured on malt agar medium at 25°C in the dark for 7 days. The colony diameter was 33mm to 40mm, the aerial hyphae were lush, light orange; the surface was flat and the texture was felt-like; the back of the colony was dark orange, and the pigment was soluble in the culture medium. Conidia were produced in large quantities, and the conidia were attached to the stalks or the top of the hyphae. They were nearly spherical, colorless, and strung, 5.6µm to 9.8µm. No sexual spore-producing structure and spores were found.

# Experimental results of stability

The contents of MK and MKA at 21 and 25 days, and the genetic stability of 3 generations are shown in Table 6.

Table 6: Contents of statins of three generations of *Monascus* M2 and mutated strains for genetic stability

| Strain property | Sample batch No. | First generation |             |                | Second generation |             |                | Third generation |             |                |
|-----------------|------------------|------------------|-------------|----------------|-------------------|-------------|----------------|------------------|-------------|----------------|
|                 |                  | MK<br>mg/g       | MKA<br>mg/g | MK+MKA<br>mg/g | MK<br>mg/g        | MKA<br>mg/g | MK+MKA<br>mg/g | MK<br>mg/g       | MKA<br>mg/g | MK+MKA<br>mg/g |
| Original        | M2               | 7.69             | 2.30        | 9.99           | 8.04              | 1.18        | 9.22           | 8.72             | 2.19        | 10.90          |
| Mutagenic       | Day 25-M2-4      | 9.06             | 2.11        | 11.16          | 10.04             | 2.56        | 12.59          | 10.02            | 3.19        | 13.21          |
|                 | Day 25-M2-5      | 9.80             | 2.48        | 12.28          | 10.13             | 2.54        | 12.67          | 9.91             | 2.94        | 12.85          |

Note: MK refers to lactone monacolin K, MKA refers to acid monacolin K, and MK+MKA refers to the total amount of monacolin K.

As shown in Table 6, the M2-4 and M2-5 strains showed a relatively high total amount of monacolin K on Day 25, and the contents of lactone monacolin K and acid monacolin K in red yeast rice after fermentation of each generation gradually increased. The total amount of monacolin K in red yeast rice after fermentation of the third generation could reach 13.21 mg/g (MK was 10.02 mg/g, MKA was 3.19 mg/g); it could be seen that the strains of the present invention showed outstanding stability in subculture. If the optimal fermentation conditions of M2-4 and M2-5 are further optimized, the content in red yeast rice would be further increased.

In the prior art, monascus strains or mutated strains have a decline in strain activity after multiple generations of subculture, especially the content of some components in the fermentation product decreases continuously. The multi-generational research findings of the present invention revealed that the strains of the present invention exhibited outstanding stability, not only addressing the issue of attenuation but also demonstrating an increase in active components.

MKA is the active ingredient of monacolin K in the human body. MK can be partially converted into MKA under certain conditions to exert its activity after entering the body. The red yeast rice fermented by the strains of the present invention not only had a high total amount, but also had a high MKA content and outstanding activity.

### Experimental Example 3: Detection of citrinin

The red yeast rice obtained after 25 days of fermentation with M7, M8, M2-4 and M2-5 of the present invention (M7 samples and M8 samples were the fifth generation samples prepared in Experimental Example 1, and M2-4 samples and M2-5 samples were the third generation samples prepared in Preparation Example 2) were taken, and detected for citrinin content by referring to the first method (immunoaffinity column purification-high performance liquid chromatography) in the national standard, GB 5009.222-2016 "Determination of Citrinin in Food".

The results are shown in Table 7 below.

Table 7: Results of citrinin content

| Strain | Citrinin content (detection limit:<br>25 $\mu$ g/kg) | Citrinin content (detection limit:<br>5 $\mu$ g/kg) |
|--------|------------------------------------------------------|-----------------------------------------------------|
| M8     | Not detected                                         | Not detected                                        |
| M7     | Not detected                                         | Not detected                                        |
| M2-4   | Not detected                                         | Not detected                                        |
| M2-5   | Not detected                                         | Not detected                                        |

The results showed that the citrinin content was not detected after detection, and the detection limit was 25µg/kg, that was,  $2.5 \times 10^{-2}$  µg/g, which was much lower than the citrinin content of 1.40-24.70 µg/g (i.e. 1400-24700 µg/Kg) in the prior art.

The citrinin content was detected by the liquid chromatography method in General Rule 0512, Part IV of Chinese Pharmacopoeia, 2020 edition. When the detection limit was 5 ppb (or 1ppm), the citrinin results were all undetectable. In addition, Figures 3 to 4 showed that the products of the present invention could be regarded as almost free of citrinin, exhibiting unexpected effects. The red yeast rice fermented by the strains of the present invention were safer.

At the detection limit of 5ppb, aflatoxin B1 was not detected. The method for detecting aflatoxin referred to the aflatoxin determination method of the pharmacopoeia (Chinese Pharmacopoeia, 2020 edition, Part IV, General Rule 2351, Liquid Chromatography/Photochemical Derivatization Method).

In addition, the study of mass spectrometry and content determination on monascus showed that the red yeast rice fermented by the three strains of the present invention contained  $\gamma$ -aminobutyric acid and ergosterol.

Although the specific embodiments of the present invention have been described in detail, those skilled in the art will understand that various modifications and substitutions can be made to those details based on all the teachings that have been disclosed, and these changes are within the scope of protection of the present invention. The full scope of the present invention is given by the appended claims and any equivalents thereof.

What is claimed is:

1. A monascus mutant strain,

which is deposited in the China General Microbiological Culture Collection Center (CGMCC), with an accession number of CGMCC No. 40403; or

which is deposited in the China General Microbiological Culture Collection Center (CGMCC), with an accession number of CGMCC No. 40487.

2. A monascus fermentation product, which is obtained by fermentation with the monascus mutant strain according to claim 1;

preferably, the monascus fermentation product is a monascus fermentation broth obtained by performing fermentation with the monascus mutant strain according to claim 1 in a liquid fermentation medium.

3. The monascus fermentation product according to claim 2, wherein,

the liquid fermentation medium comprises the following components:

30-150 g/L glucose, 2-30 g/L peptone, 0.5-7 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.01-1.2 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01-0.8 g/L  $\text{CaCl}_2$ ; prepared with potato juice or distilled water;

65-95 g/L glucose, 5-16 g/L peptone, 1-3 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.2-0.8 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05-0.2 g/L  $\text{CaCl}_2$ ; prepared with potato juice or distilled water; or

80 g/L glucose, 10 g/L peptone, 2 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.5 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g/L  $\text{CaCl}_2$ ; prepared with potato juice or distilled water to obtain the fermentation medium.

4. The monascus fermentation product according to any one of claims 2 to 3, wherein

the fermentation temperature is 25°C to 35°C, 28°C to 32°C or 30°C; and/or

the fermentation time is 20-72 hours, 24-50 hours, 36-48 hours or 48 hours.

5. A fermentation broth combination, comprising:

a yeast fermentation broth; and

the monascus fermentation broth according to any one of claims 2 to 4,

wherein the yeast fermentation broth and the monascus fermentation broth are mixed or not mixed.

6. The fermentation broth combination according to claim 5, wherein the yeast fermentation broth is a fermentation broth of *Saccharomyces cerevisiae* or a fermentation broth of beer yeast;

optionally, the yeast fermentation broth is an inactivated yeast fermentation broth or an unactivated yeast fermentation broth.

7. The fermentation broth combination according to any one of claims 5 to 6, wherein the yeast fermentation broth is obtained by a method comprising the following conditions:

a yeast is activated in a wort, preferably in a wort of 12 Brix;

the activation temperature is 25°C to 30°C, preferably 28°C; and/or

the activation time is 12-36 hours, preferably 24 hours.

8. A method for preparing red yeast rice, comprising the following steps:

inoculating the monascus mutant strain according to claim 1, the monascus fermentation product according to any one of claims 2 to 4, or the fermentation broth combination according to any one of claims 5 to 7 into a rice culture medium for cultivation to obtain a culture product, namely, red yeast rice;

optionally, further comprising a step of drying and/or crushing the culture product.

9. The method for preparing red yeast rice according to claim 8, wherein the inoculation ratio is that:

the yeast fermentation broth is 1% to 5%, 2% to 4% or 3% of the mass of the rice culture medium; and/or

the monascus fermentation broth or the fermentation broth combination is 15% to 40%, 20% to 25%, 18% to 23% or 20% of the mass of the rice culture medium.

10. The method for preparing red yeast rice according to any one of claims 8 to 9, wherein:

the rice culture medium comprises rice flour, bran, peptone, glucose and water;

preferably, expressed in mass percentage, the rice culture medium comprises the following components:

35% to 42% of rice flour, 6% to 9% of bran, 1.2% to 2.2% of peptone, 1.4% to 3.0% of glucose, and 48% to 52% of water.

11. The method for preparing red yeast rice according to any one of claims 8 to 10, characterized by any one, two or all three of the following items (1) to (3):

(1) the culture time is 15-38 days, 18-32 days, 20-25 days, 22-30 days, 24-26 days or 25 days;

(2) the culture temperature is 18°C to 35°C, 28°C to 32°C, 28°C to 30°C, or 30°C;

(3) the pH is adjusted to an appropriate value, such as a pH value of 3-7, 4-6.5 or 5-6.

12. A red yeast rice, which is prepared by the monascus mutant strain according to claim 1; preferably, which is prepared by the method for preparing red yeast rice according to any one of claims 8 to 11.

13. The red yeast rice according to claim 12, characterized in that:

it does not contain citrinin, or the content of citrinin is less than 25µg/kg or less than 5µg/kg;  
and/or

it does not contain aflatoxin B1, or aflatoxin B1 is not detected or has a content of less than 5 ppb.

14. The red yeast rice according to any one of claims 12 to 13, wherein the content of monacolin K is greater than or equal to 8.9 mg/g, greater than or equal to 9.97 mg/g, greater than or equal to 12.42 mg/g, greater than or equal to 12.85 mg/g, or greater than or equal to 13.21 mg/g.

15. The red yeast rice according to any one of claims 12 to 14, wherein the content of acidic monacolin K is greater than or equal to 1.6 mg/g, greater than or equal to 1.8 mg/g, greater than or equal to 2.0 mg/g, greater than or equal to 2.2 mg/g, greater than or equal to 2.4 mg/g, greater than or equal to 2.5 mg/g, greater than or equal to 3.0 mg/g, greater than or equal to 3.5 mg/g, greater than or equal to 3.9 mg/g, greater than or equal to 4.5 mg/g, greater than or equal to 5.2 mg/g.

16. An extract of red yeast rice, which is obtained by extracting the red yeast rice according to any one of claims 12 to 15 with ethanol or water.

17. A method for preparing monacolin K, comprising a step of separating monacolin K from the red yeast rice according to any one of claims 12 to 15 or the extract of red yeast rice according to claim 16;

preferably, the monacolin K is lactone monacolin K and/or acid monacolin K.

18. Use of the red yeast rice according to any one of claims 12 to 15 or the extract of red yeast rice according to claim 16 in the manufacture of a medicament or health food for treating or preventing hyperlipidemia or osteoporosis.

19. The red yeast rice according to any one of claims 12 to 15 or the extract of red yeast rice according to claim 16, which is used for treating or preventing hyperlipidemia or osteoporosis.

20. A method for treating or preventing hyperlipidemia or osteoporosis, comprising a step of administering to a subject in need thereof an effective amount of the red yeast rice according to any one of claims 12 to 15 or the extract of red yeast rice according to claim 16.