CHAPTER 3

EXPERIMENTAL

3.1 Materials

3.1.1 Chemicals

Acetonitrile (Merck, Germany)

Acetone (Merck, Germany)

Citrinin (Sigma, St.Louis, MO, U.S.A.)

Ethanol (Merck, Germany)

Formic acid (Merck, Germany)

Isopropanol (Merck, Germany)

Methanol (Merck, Germany)

Mevinolin (Sigma, St.Louis, MO, U.S.A.)

Phosphoric acid (Merck, Germany)

Potato Dextrose Agar (Merck, Germany)

3.1.2 Glassware

50 ml Polypropylene centrifuge tube (LP Italia, Milan, Italy)

3.1.3 Instruments

Analytical Balance (Mettler Toledo AB204-S, Switzerland)

Analytical Balance (Mettler Toledo AX205, Switzerland)

Autoclave (ALP CL-40 LDP, Japan)

Centrifuge (Heraeus Megafuge 1.0R, Germany)

High Performance Liquid Chromatography (Agilent 1100series, USA)

Incubator (Heraeus B6060, Germany)

Laminar air flow (Holten model 1.5, Denmark)

Microscope (olympus CX31RBSF Philippines)

pH meter (mettler teledo sever easy, Switzerland)

Ultrasonic bath (branson8510, USA)

UV-Visible Spectrophotometer (Agilent 8453, USA)

Rotary Evaporator (Eyela N-1000 sw, Japan)

3.1.4 Rice substrate

Chai-nat1 rice (from Bang Bo rice market, chacheongsao, Thailand)

3.2 Methods

3.2.1 Validation of Analysis methods

3.2.1.1 Development and optimization of Chromatographic conditions

A working standard solution at a concentration of 100 ppm of monacolin K was prepared in the following manner. About 1 mg of monacolin K was accurately weighed and transferred into a 10-ml volumetric flask. Adjusted to the volume with methanol and used as monacolin K stock standard solution. The chromatographic condition was optimized by testing various system conditions of mobile phase. Finally, a column of Waters Symmetry C_{18} (250mm × 4.6mm i.d., 5 µm) was chosen as the stationary phase and an isocratic elution at 1 ml/min of acetonotrile/ phosphoric acid buffer acid pH 2.5 (70:30) was applied. The analysis time was 15 min. Photo-diode array (PDA) was set at 237 nm for. The column temperature was set at 25 ◦C, and the injection volume was 20µl (modified from Ng and Shyu, 2004).

A working standard solution at a concentration of 100 ppm of citrinin was prepared in the following manner. About 1 mg of citrinin was accurately weighed and transferred into a 10-ml volumetric flask. Adjusted to the volume with methanol and used as citrinin stock standard solution. The chromatographic condition was optimized by testing various system conditions of mobile phase. Finally, a column of Waters Symmetry C_{18} (250mm × 4.6mm i.d., 5 μm) was chosen as the stationary phase and an isocratic elution at 0.75 ml/min of acetonotrile/ formic acid buffer acid pH 2.5 (70:30) was applied. The analysis time was 10 min. Photo-diode array (PDA) was set at 330 nm for. The column temperature was set at 25 ◦C, and the injection volume was 20μl (modified from Ma *et al.*, 2000).

3.2.1.2 Precision and accuracy determination

The accuracy of the method was demonstrated by determination of percent recovery. The sample blanks (dried plain rice powder) were additionally spiked with 0.025 ppm citrinin standard for testing chromatographic condition of citrinin analysis. Similarly, the sample blanks (dried plain rice powder) were additionally spiked with 0.15 ppm monacolin K standard for testing chromatographic condition of monacolin K analysis.

The sample blanks without any additional spike and those with spiked standards were prepared in triplicate. Thus, each sample detection was the average from three injections.

Relative standard deviation (%RSD) was demonstrated as the precision of analysis method in detecting the targeted metabolites in samples. Triplicate of

standards spiked in sample blanks were prepared according to the percent recovery described above.

3.2.1.3 Linearity

The calibration curves were established by plotting the peak areas against Standard concentrations using linear regression analysis. Mevinolin standards were prepared at 0, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 and 75.0 ppm and injected to HPLC/PDA with the optimum chromatographic conditions. Similarly, citrinin standards were prepared at 0, 0.05, 0.1, 0.15, 0.2, 0.4 ppm and an injected to HPLC/PDA with the optimum chromatographic conditions.

3.2.1.4 Limit of detection and limit of quantitation determination

A series of diluted standard preparations were injected with the volume of 20 µl. The limit of detection (LOD) and limit of quantitation (LOQ) were measured as the concentrations corresponding to signal-to-noise ratio of 3:1 and 10:1 respectively.

3.2.2 Preparation of red yeast rice and measurement of monacolin K and citrinin

3.2.2.1 Microorganisms

The strain of *Monascus purpureus* BCC 6131 and *Monascus ruber* TISTR 3006 were obtained from Thailand Institute of Scientific and Technological Research. Microorganisms were cultivated on potato dextrose agar (PDA) (Merck, Germany) for 10 days at 30^{o}C prior to transfer onto rice substrates.

3.2.2.2 Preparation of substrate and fermentation method

Chai-nat1 rice was purchased from local market and used as the substrate for red yeast rice production. The solid state fermentation was carried out as follows: 30 g of rice (rinsed through tap water for 5 minutes followed by drying on paper towels for 30 minutes) and 30 ml of DI water were put into 6" x 8" autoclavable polypropylene bags. The edge of polypropylene bags opening was shaped to imitate Erlenmeyer flask's neck, and was later plugged with cotton wool. The substrate was then autoclaved for 20 min at 121 °C. After being cooled down to room temperature, each bag of the substrate was inoculated with 1 cm^2 of *Monascus* culture grown on PDA plates for 10 days. The inoculated substrate was cultivated at 30^oC for 24 days. For every three days, the incubated substrate for monacolin K analysis was transferred into a plastic bag and dried in hot air oven at 50^oC for 3 days. Dried red fermented rice was finally ground into fine red fermented rice powder (modified from Pattanagul, 2007).

3.2.3 Monacolin K analysis

3.2.3.1 Extraction of monacolin K

About 1 g of red fermented rice powder was accurately weighed and transferred into a 50-ml polypropylene centrifuge tube. Samples were extracted with 8 ml of 70% methanol for 30 min on an ultrasonic bath and subsequently centrifuged for 10 min at 3000 rpm. This extract procedure was repeated three times, and the supernatants from each extraction were combined and transferred into a 25-ml volumetric flask. 70% methanol was added to make the final volume of 25 ml. The final solution was then filtered through a 0.45-µm nylon membrane filter into vials for HPLC analysis. All the samples are in triplicate sets (modified from Li *et al*. 2005).

3.2.3.2 HPLC conditions for monacolin K analysis

The optimal chromatographic conditions were established by testing various system conditions which involved stationary phase, mobile phase, injection volume and flow rate of the mobile phase. For the consideration of resolution, the column of Waters Symmetry C_{18} (250mm × 4.6mm i.d., 5 µm) was chosen as the stationary phase. The isocratic elution was applied using acetonotrile/ phosphoric buffer pH 2.5 (70:30) as the mobile phase. The amount of 20 µl sample was eluted with at 1.0 ml/min flow rate, and column temperature was controlled at 25^{o}C. The total analysis time was 15 min. The photo-diode array (PDA) detector was set at 237 nm for detection (modified from Ng and Shyu, 2004).

3.2.4 Citrinin analysis

3.2.4.1 Extraction of citrinin

About 2.5 g of red fermented rice powder was accurately weighed and transferred into a 50-ml polypropylene centrifuge tube. The triplicate sets of samples were extracted with 10 ml of methanol for 30 min on an ultrasonic bath and subsequently centrifuged for 10 min at 3000 rpm. This extract procedure was repeated twice, and the supernatants from each extraction were combined and evaporated to dryness. The residue was redissolved in 1 mL of methanol.The final solution was then filtered through a 0.45-µm nylon membrane filter into vials for HPLC analysis (modified from Ma *et al.*, 2000).

3.2.4.2 HPLC conditions for citrinin analysis

The optimal chromatographic conditions were established by testing various system conditions which involved stationary phase, mobile phase, injection volume and flow rate of the mobile phase. For the consideration of resolution, the column of Waters Symmetry C_{18} (250mm × 4.6mm i.d., 5 µm) was chosen as the stationary phase. The isocratic elution was applied using acetonitrile/ formic buffer pH 2.5 (70:30) as the mobile phase. The amount of 20 µl sample was eluted at 1.0 ml/min flow rate, and column temperature was controlled at 25°C. The total analysis time was 15 min. The photo-diode array (PDA) detector was set at 330 nm for detection.

3.2.5 Optimization of monacolin K production

3.2.5.1 Effects of Monascus strains and cultivation temperatures

Table 3.1 Selecting *Monascus* strain and cultivation temperature

experiment	*Monascus* strain	Cultivation temperature	Water added (ml)	Rice substrate	Inoculum size (cm^2)
1	*M. purpureus* BCC 6131	25°C	30 ml	30 g polished rice	1 cm^2
2	*M. purpureus* BCC 6131	30°C	30 ml	30 g polished rice	1 cm^2
3	*M. ruber* TISTR 3006	25°C	30 ml	30 g polished rice	1 cm^2
4	*M. ruber* TISTR 3006	30°C	30 ml	30 g polished rice	1 cm^2

Rice substrate was mainly prepared as described above in 3.2.2.2. Instead, to study the effect of *Monascus* strains and fermentation temperatures on monacolin K production, 1 cm^2 of either *M. ruber* TISTR 3006 and *M. purpureus* BCC 6131 culture grown on PDA were separately inoculated on substrate, and incubated either at 25°C or 30°C for 24 days as shown in table 3.1.

3.2.5.2 Effect of moisture content on monacolin K production of M. purpureus BCC 6131

Table 3.2 optimizing moisture content for monacolin K production of *M. purpureus* BCC 6131

experiment	*Monascus* strain	Cultivation temperature	Water added (ml)	Rice substrate	Inoculum size (cm^2)
1	*M. purpureus* BCC 6131	30°C	30 ml	30 g polished rice	1 cm^2
2	*M. purpureus* BCC 6131	30°C	25 ml	30 g polished rice	1 cm^2
3	*M. purpureus* BCC 6131	30°C	20 ml	30 g polished rice	1 cm^2
4	*M. purpureus* BCC 6131	30°C	15 ml	30 g polished rice	1 cm^2

Rice substrate was mainly prepared as described above in 3.2.2.2. Instead, to study the effect of moisture content on monacolin K production, 30 g of rice substrate was added with 30ml, 25 ml, 20 ml and 15 ml deionized water respectively. Inoculated substrate was incubated for 24 days.

3.2.5.3 Effect of rice substrate on monacolin K production of M. purpureus BCC 6131

Rice substrate was mainly prepared as described above in 3.2.2.2. Instead, to study the effect of rice substrate on monacolin K production of *M. purpureus* BCC 6131, 30 g of either polished rice, broken rice or unpolished rice was used as rice substrate as shown in table 3.3. The inoculated substrate was cultivated at 30°C for 24 days.

Table 3.3 selecting rice substrate for monacolin K production of *M. purpureus* BCC 6131

experiment	*Monascus* strain	Cultivation temperature	Water added (ml)	Rice substrate	Inoculum size (cm^2)
1	*M. purpureus* BCC 6131	30°C	20 ml	30 g polished rice	1 cm^2
2	*M. purpureus* BCC 6131	30°C	20 ml	30 g broken rice	1 cm^2
3	*M. purpureus* BCC 6131	30°C	20 ml	30 g unpolished rice	1 cm^2

3.2.5.4 Effect of inoculum size on monacolin K production of M. purpureus BCC 6131

Rice substrate was mainly prepared as described above in 3.2.2.2. Instead, to

study the effect of inoculum size on monacolin K production of *M. purpureus* BCC 6131, rice substrate was inoculated with 1 cm^2, 2 cm^2, 3 cm^2, 4 cm^2 and 5 cm^2 *M. purpureus* BCC 6131 culture grown on PDA plates respectively. The inoculated substrate was then cultivated at 30°C for 24 days.

Table 3.4 optimizing inoculum size on monacolin K production of *M. purpureus* BCC 6131

experiment	*Monascus* strain	Cultivation temperature	Water added (ml)	Rice substrate	Inoculum size (cm^2)
1	*M. purpureus* BCC 6131	30°C	20 ml	30 g polished rice	1 cm^2
2	*M. purpureus* BCC 6131	30°C	20 ml	30 g polished rice	2 cm^2
3	*M. purpureus* BCC 6131	30°C	20 ml	30 g polished rice	3 cm^2
4	*M. purpureus* BCC 6131	30°C	20 ml	30 g polished rice	4 cm^2
5	*M. purpureus* BCC 6131	30°C	20 ml	30 g polished rice	5 cm^2

3.2.6 Stability test

About 1 g of red fermented rice powder was accurately weighed to each 50-ml polypropylene centrifuge tube, then put into the individual required storage condition at different interval times of 0, 15, 30, 45 and 60 days. The conditions of high temperature were established in two different ovens setting at 50 and 75°C

respectively. The condition of cool storage was established in -20^{o}C freezer. At the different interval times, triplicate sets of treated samples were extracted and analyzed for percent weight variation (modified from Li *et al*. 2005).